**UNIVERSIDAD AUTÓNOMA DE MADRID**

FACULTAD DE CIENCIAS

DEPARTAMENTO DE QUÍMICA FÍSICA APLICADA

ÁREA DE CIENCIA Y TECNOLOGÍA DE LOS ALIMENTOS


# EFECTO DE LOS PARÁMETROS AGRONÓMICOS DEL VIÑEDO SOBRE LA BIODIVERSIDAD DE LEVADURAS ASOCIADAS A LA UVA

TESIS DOCTORAL

**Gustavo Adolfo Cordero Bueso**

**Madrid, 2011**

**IMIDRA**

Instituto Madrileño de Investigación y Desarrollo Rural, Agrario y Alimentario (IMIDRA)
CONSEJERÍA DE MEDIO AMBIENTE, VIVIENDA Y ORDENACIÓN DEL TERRITORIO
Comunidad de Madrid

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# A ti papá, a ti mamá

*“Si hoy todo fuera perfecto, no habría necesidad de mañana”*

# AGRADECIMIENTOS

# Agradecimientos

No quisiera empezar este trabajo sin antes agradecer sinceramente a todas esas personas que, directa o indirectamente han estado ahí siempre, los apoyos y ayudas que un trabajo de esta magnitud necesita. Yo los he recibido con creces por ello, y a riesgo de que me pueda olvidar de alguien, quisiera reflejar ciertos sentimientos que han surgido durante todo este tiempo aunque, con seguridad, lo que sea capaz de transmitir con mis palabras es tan sólo una mínima parte de lo que verdaderamente siento.

Esta Tesis va dedicada en primer lugar **a esos milagrosos microbios** capaces de transformar tan maravillosa fruta en una bebida seráfica, así como **a aquellos seres** que durante toda la Historia y gracias a su sabiduría, trabajo y esfuerzo nos han dado la posibilidad de saborear una bebida única y llena de misterios como es el vino. De esta manera comienzo brindando por **mis padres**, **Joaquín** y **Rocío**, por permitirme, llegar donde estoy sobre todo, por los consejos y esfuerzos que han tenido que realizar para que yo pueda seguir adelante una y otra vez. También por **mi hermana Rocío**, por prestarme su atención, por transmitirme cariño y por esos ánimos cuando menos lo merecía aunque era justo cuando más lo necesitaba. Os quiero.

**María José**, detrás de cada línea de esta Tesis hay una vivencia, un buen momento, una sonrisa, una caricia y un movimiento, todos ellos vinieron de ti durante todo este tiempo que hemos vivido juntos. Nuestros destinos nos separarán, pero que sepas que has dejado huella en mí, hasta el fin de los tiempos. **Marga**, si tuviera que pedir un deseo para ti, sería conocerte de nuevo, gracias por vuestra paciencia y prepararme la cena mientras escribía estas líneas.

No menos importantes son los agradecimientos que debo y quiero expresar a **mi directora**, a la Dra. Dª. **Teresa Arroyo Casado "La Tere**". Para mí, es más que mi guía y mi directora en el trabajo. Fue, por su trato y sencillez, casi una compañera más y esto se ha ido incrementando durante el tiempo que me ha dirigido, una mami. Una como pocos, en la cual sé que puedo confiar, con la que puedo hablar sin tabúes y de la que voy a obtener toda la ayuda que pueda necesitar incondicionalmente. Gracias Teresa por darme una gran dosis de confianza y por intentar inculcarme, de ese modo tan familiar y sencillo, parte de los conocimientos que posees.

Gracias, de igual modo, a la Dra. Dª. **Eva Valero Blanco** por dirigirme también desde la distancia, prestarme tu tiempo y ayuda cuando fue necesario, por preocuparte por mí y por tus consejos siempre sabios y llenos de experiencia que, sin duda, me permiten ser más eficiente y realizar más correctamente mi trabajo.

Al **IMIDRA** por brindarme la posibilidad de realizar todo este trabajo, a mis compañeros, compinches y sobre todo amigos, **Ana Serrano Somavilla**, compañera de trabajo, confidente incondicional y amiga espero que por muchos años, gracias porque te debo mucho. **Tania Balboa Lagunero** por resolver esas dudas que siempre aparecen cuando menos te lo esperas, por ser amiga mía y por contar conmigo y acompañarme a todas partes. **Javier Tello** por tu dosis de sabiduría y serenidad, por haber trabajado tanto y haber hecho que todo sea tan perfecto, y como perfecto no hay nada, para ello estaba **Irene Aporta**, para organizar, estructurar y dejarme claro qué es el orden, por esas risas espontáneas que arrancabas en mí y sobre todo por tus "besos sin gluten". Quiero agradecer a **Mariano Cabellos** los consejos que me das, hacerte llamar "padre" que sin duda lo eres de alguna manera, a **Ángel**

**Soria** siempre testigo de mis madrugadas, compartir tus alegrías y creo que eres fruto de una larga amistad y con un cariño de verdad. A **Valeria Romero** porque das el toque diferente al grupo, contándonos tus batallas y hacernos reír y a **Mar Gil**, por tu gran ayuda y combatir mi aburrimiento en mañanas grises. Además, a **Alfredo**, porque me ayudó con la Historia, que aunque sea reducida también formas parte de ella.

Grazie mille a tutti quelli che mi hanno ospitato nel Centro di Ricerca per l'Enologia di Asti, Italia, durante due meravigliosi mesi nel 2008, spezialmente a **Enrico Vaudano** ed **Emilia García Moruno**.

I would especially like to thank **Antonio García Cordente**, Dr. **Chris Curtin**, and Dr. **I. Sakkie Pretorius** from the Australian Wine Research Institute (AWRI) for hosted me at the institute during six months and for helping me with just about everything and anything, as well as being a great teachers and listener. I will miss "coffee break" at Lirra-Lirra everyday with **people from the AWRI**, as well as those who I lived or I met during my living in Australia, especially to **Ben Williams** and **Martin Curnow**. Their quiet encouragement and pride in achievements have always been motivation to succeed.

No me olvido, en absoluto, de mis **AMIGOS** de siempre y ahora, de mis **compañeros de facultad**. Les estoy agradecido por el apoyo recibido en todo, porque ellos me han ayudado también, en cierto modo, a llegar hasta aquí. No cambiéis chicos, seguid así. Gracias, en definitiva, **A TODOS** los que han hecho posible este trabajo con sus consejos, apoyos, ánimos... y a aquellos que, simplemente con estar ahí, me enriquecen como persona y me ayudan a seguir mirando hacia adelante.

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# Índice

# Capítulo 1

# PLANTEAMIENTO DE LA TESIS

Capítulo 1

# 1. Planteamiento de la Tesis

Esta Tesis doctoral se ha realizado gracias al desarrollo de una beca de formación del personal investigador del Instituto Madrileño de Investigación y Desarrollo Rural, Agrario y Alimentario (**IMIDRA**) perteneciente a la Consejería de Medio Ambiente, Vivienda y Ordenación del Territorio de la Comunidad de Madrid, en el periodo comprendido entre los años 2007 - 2011 y está basada en los resultados obtenidos en los proyectos de investigación *IMIDRA FP07-AL2 "Efecto de los parámetros agronómicos sobre la microbiota del viñedo de la Comunidad de Madrid"* y *FP-IA-07 "Caracterización de levaduras autóctonas de la Comunidad de Madrid"*, del IMIDRA.

## 1.1. Objetivos de la Tesis

El presente trabajo se propuso como objetivo fundamental, conocer y evaluar cómo afectan distintas prácticas agronómicas de manejo del viñedo sobre la microbiota de levaduras de interés enológico asociadas a la uva.

Los objetivos específicos fueron los siguientes:

1. Estudiar de la influencia del sistema de defensa biológica, ecológico o convencional, sobre la microbiota asociada a la uva en el viñedo.

2. Valorar de la influencia de la variedad de vid (Garnacha, Barbera y Syrah) sobre la microbiota asociada a la uva.

3. Analizar la influencia de las distintas estrategias de manejo del suelo del viñedo (suelo desnudo por herbicidas, suelo desnudo por laboreo y suelo con cubierta vegetal) en la microbiota asociada a la uva.

4. Estudiar la influencia de distintos fungicidas y sus dosis de aplicación (azufre y penconazol) sobre la microbiota asociada a la uva.

5. Evaluar la influencia de algunas prácticas enológicas como la utilización de levaduras comerciales, mediante el estudio de la permanencia y evolución de una levadura comercial en el viñedo diseminada voluntariamente.

Por otra parte, este trabajo pretendió contribuir al crecimiento de la colección de levaduras autóctonas de la Comunidad de Madrid, constituyendo un valioso recurso biotecnológico para la industria enológica y alimentaria.

## 1.2. Producción científica de la Tesis

Esta sección pretende mostrar una recopilación ordenada de la producción científica a la que ha dado lugar estos años de experimentación, quedando recogida en las siguientes publicaciones:

**Cordero-Bueso, G.,** Arroyo, T., Serrano, A., Tello, J., Aporta, I., Vélez, M.D., Valero, E. (2011) ***Influence of the farming system and vine variety on yeast communities associated with grape-berries***. International Journal of Food Microbiology 145, 132-139.

**Cordero-Bueso, G.,** Arroyo, T., Serrano, A., Valero, E. (2011) ***Influence of different floor management strategies of the vineyard on the***

***natural yeast population associated with grape berries***. International Journal of Food Microbiology 148, 23-29.

**Cordero-Bueso, G.,** Arroyo, T., Serrano, A.,Valero, E. (2011) ***Effect of the antifungal sulfur and penconazole on indigenous yeast populations associated with grape berries***. *Food Control. Submitted.*

**Cordero-Bueso, G.,** Arroyo, T., Serrano, A.,Valero, E. (2011) ***Remanence and Survival of commercial yeast in different ecological niches of the vineyard***. FEMS Microbiology Ecology., 77 (2), 429-437.

**Cordero-Bueso G.,** Valero E., Serrano A., Arroyo T. (2010). ***Resultados preliminares del efecto de parámetros agronómicos sobre la microbiota del viñedo en la comunidad de Madrid.*** En: Reuniones del grupo de trabajo de experimentación en viticultura y enología, 24ª reunión. Edita: Ministerio de Medio Ambiente y Medio Rural y Marino. Secretaría General Técnica, centro de publicaciones. Madrid (España), pp. 207-213.

Arroyo, T., **Cordero-Bueso, G.**, Serrano, A., Valero E. (2010). ***β-glucosidase production by non-Saccharomyces yeast isolated from vineyard***. In: Blank, I., Wüst, M., Yeretzian, C. (Ed.), Expression of Multidisciplinary Flavour Science, Winterthur (Switzerland), pp. 359-362.

Cabe destacar que este trabajo también ha dado lugar a numerosos pósters (12) y comunicaciones orales (3) en congresos tanto nacionales como internacionales, así como presentaciones en simposios y *proceedings* de congresos (7).

# Capítulo 2

# INTRODUCCIÓN GENERAL

# Capítulo 2

# 2. Introducción general

**"*We continue to live out our past by drinking wine made from a plant that has its origins in the ancient Near East...*"**

- Quote by Dr. Patrick McGovern in Penn Museum, Philadelphia, USA (2011), *The University of Pennsylvania museum of Archaeology and Anthropology: http://www.penn.museum*

## 2.1. La vid y el vino de ayer, la Historia de hoy

La fermentación deliberada de hidratos de carbono en alcohol (etanol) ha sido reconocida como una de las numerosas innovaciones que marcan la transición desde el Paleolítico a las sociedades Neolíticas. Algunos autores creen que el conocimiento del proceso de fermentación fue al menos uno de los factores que motivaron a la domesticación de plantas silvestres (cereales, olivo, vid, etc…) y el desarrollo de la tecnología cerámica (Vitelli, 1989; McGovern, 2007, 2009; Barnard et al. 2010).

La primera evidencia inequívoca de la vinificación intencional aparece en unas excavaciones arqueológicas en el complejo de cuevas Areni-1, en el sureste de Armenia, dónde en el año 2010 se revelaron instalaciones y artefactos con una datación de alrededor de 4000 a.C. y en buen estado de conservación (Barnard et al., 2010). También se han encontrado residuos de vino en ánforas claramente indicadas en muchas tumbas del antiguo Egipto. Existen pruebas, tanto para vinos blancos como tintos en las ánforas que se encuentran en la tumba del faraón Tutankamón (1325 a.C) (Guasch–Jané et al., 2004, 2006).

Por otro lado, se piensa que la domesticación de la uva de vinificación (*Vitis vinifera*) se produjo en la misma zona, también se han encontrado restos de lo que parecen ser viñas domésticas en un poblado neolítico en Georgia (Ramishvili, 1983). Es en estas regiones dónde la distribución natural de *V. vinifera* se aproxima más al origen probable de la agricultura occidental (Zohary y Hopf, 2000). Paralelamente, la domesticación de la vid también pudo haber ocurrido de forma independiente en España (Rivera-Núñez y Walker, 1989). Los primeros indicios de la aparición de la especie *Vitis vinifera sylvestris* L. datan en 50 millones de años, no obstante, gracias a la Paleobotánica, se piensa que su especie ancestral pertenece al género *Ampelopsis*, arbustos trepadores de los cuales se han encontrado trazas que indican su existencia desde hace 500 millones de años (McGovern, 2003).

La fecha de llegada de la vitivinicultura a la Antigua Grecia es aún incierta. Muchos autores creen que los primeros viticultores griegos son los minoicos, civilización pre-helénica procedentes de la isla de Creta, gracias al descubrimiento de un recipiente de piedra utilizado para el pisado de la uva con una datación de 1600 a.C. en la localidad de Vathipetro, Creta (Roubelakis-Angelakis, 2009). Así, se puede decir que la Antigua Grecia fue pionera en vivir la primera “Edad de Oro” del vino hasta la toma de Grecia por parte del imperio Romano en el año 146 a.C. (Fleming, 2001).

La viña continuó su expansión a medida que los romanos conquistaban nuevos territorios, introduciéndose en el sur de Gran Bretaña, Portugal, Rumanía, Alemania y norte de Francia. Los romanos adoptaron nuevas tecnologías para la mejora de la viticultura, sistemas de riego, orientación en laderas y propagación de la vid, y vinicultura, como la introducción de barricas de madera en lugar de tinajas de barro

o el sellado con puzolana (arcilla volcánica) y posteriormente con corcho, para garantizar una mejor conservación (Jackson, 2009).

Con la caída del Imperio Romano, la viticultura descendió gradualmente debido al control feudal y la mala gestión de los viñedos resultando en una producción baja y vinos de mala calidad. Entonces fueron los monasterios quienes adquirieron los viñedos y bodegas manteniendo la tradición e incluso haciendo mejoras. Así, por ejemplo, los monjes cistercienses de Borgoña (Francia) fueron los primeros en estudiar el suelo de la Côte d'Or y seleccionaron las cepas más aptas para el terreno, rodeando el viñedo con un muro. Además, experimentaron con la poda y en la elección de parcelas no expuestas a heladas para obtener una madurez en las uvas óptimas (Dominé, 2005).

El vino comenzó a tomar su expresión moderna en el siglo XVII. El uso de azufre en el tratamiento del barril parece haber llegado a ser bastante común en Europa occidental en esta época. Esto habría aumentado considerablemente la probabilidad de producir vinos de mejor calidad y ampliar su potencial de envejecimiento (Dominé, 2005). La estabilidad de los vinos dulces capaces de permanecer intactos durante décadas o siglos, también comenzaron a aparecer a mediados de los años 1600, siendo pioneros los productores húngaros con los vinos de Tökaj (Lambert-Gocs, 2010).

En 1860 apareció por primera vez en el Midi francés la más devastadora de las plagas, la filoxera de la vid (*Dactylosphaera vitifoliae*), que causó enormes pérdidas en toda Europa. Hacia 1880 se comenzó a plantar cepas europeas sobre cepas americanas (*Vitis labrusca*) mediante portainjerto con el fin de salvaguardar la vid europea de los posibles ataques de la filoxera, ya que la vid americana es resistente a ella. Otras enfermedades causadas por hongos fitopató-genos como el

oídio (*Uncinula necator*) o el mildiu (*Plasmopara vitícola*) fueron un grave problema en la época debido a la introducción de la vid americana en Europa. A raíz de estos problemas emergió el desarrollo de técnicas de viticultura y pesticidas. Los esfuerzos llevados a cabo para superar las consecuencias de la filoxera y las crisis económicas incluyeron el desarrollo de la legislación vitícola, intentando además combatir el fraude: vinos ordinarios etiquetados bajo grandes nombres, vinos adulterados, etc. De esta forma nació el sistema francés de denominaciones de origen (AOC) y las reglamentaciones que se han inspirado en él, aunque sea parcialmente, en casi todo el Mundo. Hoy, variedades, límites territoriales, métodos de poda, uso de productos fitosanitarios, etc… está reglamentado (Dominé, 2005).

El descubrimiento de Pasteur, en la década de 1860, sobre la importancia central de las levaduras y las bacterias responsables de la fermentación alcohólica ha puesto en marcha una cadena de acontecimientos que ha producido un increíble avance en la viticultura y enología. La ciencia empezó entonces a desempeñar un papel importante y se desarrollaron programas de investigación sobre la vid, la fermentación o la crianza en bodega. Entre ellos, estudios de ecología de levaduras en fermentaciones, bodegas y viñedos, así como los factores por los que se ven afectados, tales como la edad o el tamaño del viñedo, el tipo de suelo, la utilización de fungicidas, etc. (Pretorius, 2000), muchos de los cuales no han sido determinados con precisión.

## 2.2. Levaduras: origen, importancia y uso

Los términos levadura y fermentación provienen etimológicamente de las palabras “hervir” o “burbujear” haciendo referencia al aspecto del mosto de vino durante la fase tumultuosa de la fermentación, cuando el

azúcar se convierte bioquímicamente en etanol y dióxido de carbono, pero la fermentación producida por las levaduras es mucho más que eso. De hecho, es la responsable de la mayoría de los cambios asociados con la biotransformación del mosto en vino. El aroma, el sabor, la sensación en el paladar, el color y la complejidad química son producidos en su mayor parte por la levadura, conforme diversifica y amplía su mundo con los productos de su metabolismo.

La evidencia anterior de la conexión entre el vino y *Saccharomyces cerevisiae* proviene de un ánfora encontrada en la tumba del rey Escorpión, durante la dinastía de Narmer (1350 a.C.). *S. cerevisiae* fue confirmada por la extracción de ADN de una de las ánforas y demostró que guardaba más similitud con cepas modernas de *S. cerevisiae* que con otras especies estrechamente relacionadas como *Saccharomyces bayanus* y *Saccharomyces paradoxus*, esta última considerada progenitora de *S. cerevisiae* (Cavalieri et al., 2003). Otras levaduras presentes en las uvas, como *Kloeckera apiculata* y varias especies del género *Candida* fácilmente pueden iniciar la fermentación, sin embargo, rara vez la completan dada la sensibilidad al alcohol acumulado y su baja capacidad fermentativa (Fleet, 2008; Heard y Fleet, 1988; Romancino, 2010). Por el contrario, la cerveza con un contenido de alcohol inferior puede haber sido inicialmente fermentada por una levadura diferente a *S. cerevisiae* (Jackson, 2009).

Por otra parte, el origen de *S. cerevisiae* es a veces controvertido, unos autores defienden que en el viñedo hay suficientes levaduras como para llevar a cabo la fermentación (Pretorius, 2000, Valero et al., 2007, Cordero-Bueso et al., 2011) y que la presencia o ausencia de *S. cerevisiae* en el mismo depende de cada cepa de vid y de cada racimo de uvas (Török et al., 1996; Pretorius, 2000), otros consideran que *S.*

*cerevisiae* proviene de la microbiota residente en la bodega (Fleet y Heard, 1993; Vaughan - Martini A., 1995; Martini A., 1997). En el viñedo, las levaduras pueden ser transportadas del suelo a las uvas por insectos o por el viento (Pretorius, 2000; Francesca et al., 2010; Goddard et al., 2010). Especies fermentativas de *Saccharomyces* están presentes en muy bajo número en las uvas, siendo los microorganismos predominantes las levaduras apiculadas y otras especies oxidativas (Fleet y Heard, 1993; Valero et al., 2007). Mortimer y Polsinelli (1999) observaron que las bayas de uva dañadas eran ricos reservorios de *S. cerevisiae* en el viñedo, que podría constituir una reserva natural de *S. cerevisiae*. Según esto, consideramos de un enorme interés la obtención de datos que permitan conocer la influencia de las prácticas agronómicas sobre la biodiversidad de la microbiota de levaduras asociada a la uva.

Inicialmente, la producción de vino se llevaba a cabo partiendo de la microbiota natural por fermentaciones espontáneas. Las levaduras autóctonas que se encuentran en la piel de la uva y la microbiota asociada al ambiente de las bodegas, son las responsables del proceso fermentativo. Las levaduras presentes en los viñedos, alcanzan la uva debido a la diseminación del viento e insectos (Lafon-Lafourcade, 1983; Pretorius, 2000; Valero et al. 2005), esta microbiota puede verse afectada por un gran número de factores entre los que cabe destacar la temperatura, la pluviosidad, la altitud, el grado de madurez de la uva y el uso de fungicidas (Boulton et al., 1996; Fleet et al., 2002; Valero et al., 2005; Raspor et al., 2006; Chavan et al., 2009; Li et al., 2010; Cordero-Bueso et al., 2011). Una vez en las bodegas, las diferentes condiciones que se dan en el mosto a lo largo de la fermentación alcohólica, provoca una sucesión de distintas especies de estas levaduras, según su adaptación a las mismas. Las especies predominantes en las primeras etapas de la fermentación son las del género *Hanseniaspora (*anamorfo

*Kloeckera)* y *Candida,* seguidas de algunas especies de *Metschnikowia* y *Pichia* en las etapas intermedias, cuando el etanol producido alcanza el 3-4% v/v. Las últimas etapas de la fermentación natural del vino están dominadas por cepas tolerantes al alcohol, mayoritariamente pertenecientes a la especie *Saccharomyces cerevisiae* (Ribéreau-Gayon et al., 2000; Pretorius, 2000; Di Maro et al., 2007; Zott et al., 2008). Otros géneros como *Brettanomyces*, *Cryptococcus*, *Issatchenkia*, *Kluyveromyces*, *Rhodotorula*, *Schizosaccharomyces, Torulaspora* y *Zygosaccharomyces* pueden estar presentes durante la fermentación y consecuentemente en el vino, algunos de los cuales son capaces de alterar posteriormente la calidad sensorial del mismo (Fleet y Heard, 1993; Suárez-Lepe, 1997; Ribéreau-Gayon et al., 2000; Pretorius, 2000; Mills et al., 2002; Di Maro et al., 2007; Zott et al., 2008). La microbiota presente en las superficies de las bodegas, está representada mayoritariamente por *S. cerevisiae* (Martini y Vaughan-Martini, 1990; Fleet y Heard, 1993; Suárez-Lepe, 1997; Valero et al., 2007; Rodríguez et al., 2011) aunque también han sido aisladas en este ambiente especies de los géneros *Kloeckera*, *Torulaspora*, *Brettanomyces*, *Candida*, *Hansenula* y *Pichia*.

Las levaduras no-*Saccharomyces* secretan una serie de enzimas (esterasas, lipasas, ß-glucosidasas, proteasas, xilanasas, celulasas, etc.) (Fernández et al., 2000; Mendes Ferreira et al., 2001; Strauss et al., 2001; Arroyo et al., 2010), que pueden interaccionar con los sustratos presentes en el medio, mejorando algunas etapas del proceso como son la maceración, filtración y clarificación, el incremento de rendimiento, la extracción del color, etc, aumentando así el potencial aromático y organoléptico del vino (Suárez-Lepe, 1997; Schödl, 2002; Marais, 2003; Villimburgo, 2003; Romano et al., 2003; Salinas et al., 2003; Gómez-Mínguez et al., 2007; Zott et al.,

2008). La industria enológica está mostrando un gran interés por levaduras presentes en las primeras etapas de las fermentaciones espontáneas, no pertenecientes al género *Saccharomyces*, debido a que aportan al vino determinados compuestos beneficiosos para el aroma y tipicidad de los vinos de una región dada (Ferraro et al., 2000; Mesa et al., 2000; Rojas et al., 2003; Povhe et al., 2005; Rodríguez et al., 2010, 2011).

A partir de los años 80, la utilización de las levaduras secas activas como cultivos iniciadores de la fermentación se ha ido extendiendo considerablemente. Hoy, la mayoría de la producción del vino está basada en el uso de levaduras comerciales como cultivos iniciadores de la fermentación, las cuales han sido aisladas de viñedos o bodegas y seleccionadas por sus buenas propiedades fermentativas o por cualquier otra propiedad de interés para la elaboración del vino. Esta práctica trata de asegurar una fermentación rápida, fiable y homogénea, reduciendo el riesgo de enlentecimientos o paradas de fermentación, así como de contaminaciones microbianas con las consecuentes pérdidas en la calidad organoléptica y posibles variaciones en el producto final, provocadas por levaduras autóctonas (Lambrechts y Pretorius, 2000; Romano et al., 2003; Valero et al., 2005; Viana et al., 2008). No obstante, su uso continuado conlleva a una colonización de las bodegas donde son utilizadas, con la consecuente disminución de la biodiversidad microbiana de las mismas. Debido a esto, nuestro grupo de investigación ha empezado a mirar de nuevo hacia el viñedo como una posible fuente para la recuperación y preservación de la microbiota autóctona, en parte comprometida como consecuencia de una masiva utilización de levaduras seleccionadas. Por este motivo, el viñedo constituye hoy día el hábitat ideal donde se debe preservar e incluso favorecer la presencia de especies fermentativas de levaduras. Además, actualmente hay un

creciente interés tanto por cepas autóctonas de *S. cerevisiae* como por otras levaduras autóctonas que pueden contribuir al conjunto de caracteres sensoriales de los vinos, incluso en fermentaciones dirigidas, así como por el uso de cepas autóctonas de *S. cerevisiae* en cultivos iniciadores mixtos, dirigidos a reflejar la biodiversidad de una región determinada. De este modo, han sido realizados extensos estudios ecológicos tanto en bodegas (Querol et al.,1992a, 1992b; Schütz y Gafner,1993; Martínez et al., 1995; Gutierrez et al., 1997; Constantí et al., 1998; Arroyo, 2000; Sabaté et al., 2002; Esteve-Zarzoso et al., 2004; Legras et al., 2007; Viana et al., 2008; Stringini et al., 2008; Rodríguez et al., 2011) como en viñedos (Frezier y Dudourdieu,1991; Vezinhet et al.,1992; Querol et al., 1994; Briones et al., 1996; Sabaté et al., 1998; Ganga y Martínez, 2004; Schuller et al., 2005; Valero et al., 2005, 2007; Stringini et al., 2008; Cordero-Bueso et al., 2011), utilizando métodos moleculares de identificación, con el propósito de seleccionar nuevas levaduras mejor adaptadas a condiciones de fermentaciones locales (Pretorius et al., 1999; Van der Westhuizen et al., 2000; Khan et al., 2000; Rodríguez et al., 2011). Éstos y otros trabajos (Versavaud et al., 1995; Torija et al., 2001, 2003; Lopes et al., 2002; Esteve - Zarzoso et al., 2004; Schuller et al., 2004; Suárez Valles, 2006; Zott et al., 2008), muestran una gran diversidad de patrones genéticos entre la microbiota fermentativa presente en el viñedo.

## 2.3. Influencia de los parámetros agronómicos sobre las levaduras

Cuando se estudia la biodiversidad de levaduras en el viñedo, debemos tener en consideración la influencia de numerosos factores algunos de ellos conocidos, como son las condiciones climatológicas y el

estado de madurez de la uva (Hierro et al., 2006; Raspor et al., 2006). Así, algunos autores señalan que en los años o en zonas de mayor pluviosidad, se obtienen mostos de menor contenido en azúcar, provocando unas fermentaciones más lentas debido a una menor proporción de levaduras del género *Saccharomyces* (Longo et al., 1991; Angulo et al.; 1993; Ganga y Martínez; 2004). Aunque se sabe que diversos factores ligados al viñedo, como son la edad, el tamaño, el tipo de suelo, la utilización de fungicidas, etc. (Pretorius, 2000; Fleet, 2008; Cordero-Bueso et al., 2011) influyen sobre la diversidad de las poblaciones autóctonas de levaduras, otros factores como la variedad de vid, los sistemas de manejo del suelo, los sistemas de producción agrícola o los tipos y dosis de fungicidas utilizados, no han sido estudiados en profundidad hasta el momento.

Además aún existen dudas sobre los estudios de biodiversidad de levaduras en enología y viticultura, son muchas las preguntas que necesitan una respuesta; ¿El desarrollo de la fermentación depende de la levadura empleada o del propio substrato? ¿Pueden diferentes cepas de levaduras dar como resultado un producto final de similares características? ¿Qué biodiversidad de levaduras está realmente implicada en la fermentación alcohólica?. Un mayor conocimiento de la ecología de levaduras asociadas a la uva podría aportar la información suficiente para resolver este tipo de cuestiones, además de tener consecuencias directas en la elaboración del vino. Son numerosas las técnicas de Biología Molecular utilizadas para la identificación de diferentes especies de levaduras y consecuentemente completar estudios de ecología, pero la biodiversidad no depende sólo de la riqueza de especies, sino también de la dominancia relativa y la abundancia de cada una de ellas. Las especies, en general, se distribuyen según jerarquías de abundancias, desde algunas especies

muy abundantes hasta algunas muy raras. Cuanto mayor es el grado de dominancia de algunas especies y de rareza de las demás, menor es la biodiversidad de la comunidad (Moreno, 2001; Cordero-Bueso et al., 2011a). Para ello existen potentes herramientas estadísticas muy utilizadas en Ecología clásica y de microorganismos, como son los índices de biodiversidad de Shannon-Wiener o Simpson. Estos índices se caracterizan por ser fáciles de calcular y relativamente sencillos de interpretar, además de poseer una larga historia en su aplicación en Ecología.

### *2.3.1. Gestión del suelo*

El manejo del suelo comprende el conjunto de operaciones de cultivo que se realizan en el mismo, con objeto de lograr un desarrollo satisfactorio de la vid, actuando sobre sus componentes físico-químicos y biológicos, sin menoscabar su potencial de producción (Hidalgo, 1999). Se han realizado numerosos trabajos en los distintos tipos de manejo del suelo en viñedos, pero hasta el momento sólo se ha tenido en cuenta la macro y microbiota asociada al mismo. En lo que respecta a las poblaciones de levaduras presentes en la piel de la uva, son escasos los datos sobre cómo les puede afectar el tipo de manejo del suelo.

#### *2.3.1.1. Laboreo del suelo*

El laboreo es la práctica más extendida y antigua en el cultivo de la vid. Sobre todo en regiones secas y cálidas, donde la disponibilidad de agua es limitada. Las principales razones por las que se lleva a cabo esta práctica son destruir las malas hierbas, airear la tierra y conservar la humedad. Otras muchas serían mejorar la penetración del agua de lluvia, la estructura del suelo, mantener reservas de humedad, regular la

temperatura, incrementar la intensidad de reacciones químicas y bioquímicas, etc… (Hidalgo, 1999; Pastor et al., 2001). Contrariamente las labores pueden tener efectos desfavorables para el viñedo la formación de la "suela de labor" provocada por el paso de la maquinaria agrícola, difusión de parásitos, mutilación de raíces en casos desfavorables, agravación de clorosis, incremento en la erosión de suelos en pendiente, riesgo de corrimiento, etc. (Hidalgo, 1999; White, 2009).

Una gran variedad de organismos de los diferentes reinos animal, vegetal, algas, hongos y bacterias viven de forma natural en el suelo. De acuerdo a sus necesidades nutritivas, unos los podemos encontrar en capas más profundas del suelo del viñedo y otros en la superficie. Algunos se pueden observar a simple vista como son los nematodos, insectos, pequeños mamíferos, etc… pero otros no, como es el caso de algunos microorganismos como los hongos (incluídos ascmicetos), bacterias o algas. Conceptualmente, podemos reconocer cierta jerarquía en cuanto a cadenas tróficas se refiere, los diferentes niveles se organizan según los nutrientes que cada organismo emplee para su supervivencia. Estos nutrientes están formados por materia orgánica procedente de los depósitos de hojas, raíces muertas, exudados o restos de otros organismos (White, 2009). Algunas levaduras son ascomicetos heterótrofos capaces de degradar hemicelulosa, lignina y quitina y con ello pueden vivir en el suelo, pero las poblaciones de levaduras no son significativas en cuanto a número de individuos en comparación con otros microorganismos presentes en el suelo (Angle, 2000; Thorn, 2000). Es más fácil encontrar levaduras en la vid propiamente dicha, especialmente en las hojas y uvas (Cordero-Bueso et al., 2011b, 2011c) puesto que la fuente principal de carbono presente en la uva la conforma

la glucosa, y es mucho más fácil de degradar por la mayoría de las especies de levadura.

Son numerosos los estudios llevados a cabo en suelos para evaluar la diversidad de microorganismos y ver cómo afecta el laboreo a los mismos o viceversa (Angle, 2000; Thorn, 2000; White, 2009), pero no si el laboreo afecta de manera directa o indirecta a las levaduras colonizadoras de la vid. Sería necesario profundizar en ello con el fin de obtener una información válida que ayudara a los viticultores a decidir qué estrategia utilizar para el cultivo de la vid.

#### *2.3.1.2. Cubiertas vegetales*

La alta pluviometría en periodos secos de algunas regiones vitivinícolas del Mundo permite el establecimiento de una cubierta vegetal permanente en el viñedo, manteniendo las líneas de cepas libres por medio de herbicidas. Sin embargo, en áreas semiáridas o áridas las cubiertas vegetales sólo se recomiendan en periodos húmedos y nunca en periodos de escasez de lluvias y altas temperaturas, ya que se crea cierta competencia por el agua entre la vid y las hierbas que conforman la propia cubierta (Hidalgo, 1999; Tesic et al., 2007).

El uso de cubiertas vegetales se ha descrito como una alternativa sostenible de manejo del suelo que presenta múltiples ventajas. Las cubiertas vegetales aportan un alto contenido de materia orgánica y de nutrientes en el suelo, debido a la degradación de la biomasa aérea y subterránea; mejorar las propiedades físicas como porosidad, estructura, y estabilidad de los agregados; incrementar la capacidad de retención de humedad y la capacidad de intercambio catiónico del suelo; reducir el escurrimiento del agua y evitar la erosión y aumenta la actividad

biológica en el suelo (Frye y Blevins, 1989; Hidalgo, 1999; White, 2009). Ventajas adicionales en el caso de la vid, son contribuir a la disminución de la población de malezas de difícil control y permitir el control de algunas especies de nematodos perjudiciales (Aballay y Insunza, 2002). Además, diversos trabajos experimentales han demostrado que el manejo inapropiado del suelo afecta a las características físico-químicas del mismo, lo cual incide a la productividad de la viña y en la composición del mosto (Kliewer, 1991; Sicher et al., 1995; Murisier y Zufferey, 1997; Murisier et al., 1999; Ovalle et al., 2007; Marques et al., 2010).

Por otro lado, se debe tener en cuenta el color del suelo, ya que este influye significativamente en el crecimiento y vigor de la vid, así como en la maduración de las uvas, alterando su composición en azúcar, antocianos, polifenoles y contenido en aminoácidos libres (Robin et al., 1996; Jackson, 2009). Los suelos oscuros o con cubierta vegetal absorben el calor durante el día y lo liberan por la noche, sin embargo los suelos cálcicos o desnudos son más claros y durante el día se refleja la luz y no almacenan el calor necesario que posteriormente sería liberado en horas sin luz. De este modo, el albedo influye sobre las poblaciones de levaduras asociadas al viñedo de manera directa al incidir en la madurez de la uva y su correspondiente contenido en sustancias nutricionales (Robin et al., 1996; White, 2009).

### *2.3.2. Empleo de pesticidas*

La palabra “pesticida” hace referencia a todos aquéllos compuestos químicos, tanto orgánicos como inorgánicos tales como miticidas, herbicidas, fungicidas, reguladores del crecimiento, nematicidas, insecticidas, etc… que están destinados a salvaguardar a los cultivos de

enfermedades, plagas o infecciones por diversos vectores (Lee, 1990). Uno de los principales problemas en viticultura es la susceptibilidad de la vid a infecciones por hongos como la podredumbre blanca y gris, el oídio y el mildiu. A estos efectos, es necesaria la aplicación de productos capaces de combatir dichas micosis. Se da por hecho de que los fungicidas son los productos más eficaces para ello, pero ¿Se ven afectados otros microorganismos?. Es obvio que son productos químicos, que se acumulan en el suelo, que pueden ser infiltrados y transportados por el agua hacia acuíferos, contaminándolos y poniendo en riesgo la salud de muchos seres vivos, así como la vida útil de las aguas subterráneas. Pero hay que tener en cuenta que en dosis adecuadas pueden ser beneficiosos y eliminados por la propia naturaleza mediante la incorporación de los mismos en los ciclos biogeoquímicos (White, 2009). El importante avance tecnológico en el desarrollo de nuevos productos más inocuos contra otros seres vivos distintos al organismo diana y más "respetuosos" con el medio natural avanza cada día. En nuestro estudio se ha pretendido saber qué tipo de fungicida es menos perjudicial, y en qué dosis para las poblaciones de levaduras presentes en la uva.

#### *2.3.2.1. Herbicidas*

El empleo de herbicidas para el control de las malas hierbas evita en gran parte los efectos desfavorables para el suelo que puede ocasionar el laboreo, además reduce su coste económico tanto de mano de obra como de tracción, permitiendo el empleo de este tiempo para otras labores como la poda, operaciones en verde, tratamientos (Hidalgo, 1999). El empleo de herbicidas además mejora la estructura del suelo, evolucionando favorablemente la materia orgánica de las capas superficiales, reduciendo la erosión en terrenos de poca pendiente,

disminuyendo el riesgo de heladas primaverales y de daños causados por la maquinaria agrícola, etc. (Hidalgo, 1999; Busse et al., 2001). Además, existen estudios que demuestran que la aplicación de herbicidas en dosis adecuadas no tienen efectos negativos sobre la macro y microbiota del suelo, más bien son beneficiosas dado que pueden estimular las poblaciones de microorganismos presentes en el suelo debido a un aporte extra de nutrientes (Araújo et al., 2003; Krzysko-Lupicka y Sudol, 2008). Por el contrario, conlleva posibles riesgos de fitotoxicidad, por lo que es necesario una precisa dosificación y adecuada aplicación de los herbicidas por parte del viticultor. Además el efecto del estos productos químicos dependen del tipo de suelo y de la naturaleza de las malas hierbas (Hidalgo, 1999).

Uno de los herbicidas más comunes junto al Diurón es el Glifosato debido a su rápida inactivación en el suelo y a su baja toxicidad. Son numerosos los estudios realizados en laboratorio sobre la posible influencia negativa de estos productos sobre los diferentes microorganismos presentes en el viñedo (Santos y Flores, 1995; Krzysko-Lupicka y Orlik, 1997; Busse et al., 2001; Krzysko-Lupicka y Sudol, 2008), pero son muy escasos los experimentos que intentan evaluar sus efectos en campo abierto. Así, una evaluación más precisa de la influencia de los herbicidas sobre la microbiota de levaduras asociadas a la uva, es necesaria.

#### *2.3.2.2. Fungicidas*

Con respecto a la utilización de fungicidas, Van der Westhuizen et al. (2000) observaron que en años en los que las condiciones climáticas eran adversas, se observaba una severa infección de hongos, haciendo necesaria una fuerte aplicación de productos químicos repetidas veces y

en diferentes estados de desarrollo de la vid. En estas condiciones se vio que la proporción del número de cepas de *S. cerevisiae* se redujo drásticamente. Muchos fungicidas actúan directamente sobre la división celular, respiración y biosíntesis de esteroles de los hongos (Leroux, 2003). Esto puede incidir además sobre los diversos géneros de levaduras presentes en el viñedo. Por esta razón, cada vez son más específicos los fungicidas presentes en el mercado contra el parásito a tratar. Pero es necesario justificar su uso y si realmente son tan efectivos sobre esos parásitos o también afectan a otros microorganismos o a la salud humana (Thomson y Hoffmann, 2007). Por otro lado, los viticultores que se dedican a la producción ecológica no tienen permitido el uso de fungicidas sintéticos, pero sí aquellos derivados del azufre o cobre, que se consideran más respetuosos con el medio ambiente (siempre en dosis recomendadas) ya que se pueden incorporar a los diferentes ciclos biogeoquímicos de manera natural (Comitini y Ciani, 2008; White, 2009). Estudios recientes confirman que los productos fitosanitarios afectan a la biodiversidad de levaduras presentes en el viñedo (Ribeiro et al., 2000; Komárek et al., 2010; Cordero-Bueso et al., 2011a, 2011b), pero los fungicidas son necesarios para evitar pérdidas en cosechas y su consecuente valor económico. De este modo, estudios para aportar información, así como recomendar al viticultor sobre el uso adecuado de fungicidas, en qué dosis y qué productos aplicar, son de gran importancia.

## 2.4. Supervivencia y permanencia de una levadura comercial en el viñedo

Como resultado de las prácticas enológicas modernas y la diversificación de los diferentes vinos, existe un enorme interés por la búsqueda de nuevas cepas de levaduras vínicas para obtener un

producto de alta calidad. Durante las últimas tres décadas, el mejor conocimiento de la fisiología y la genética de *S. cerevisiae* ha permitido el desarrollo de nuevas herramientas de biología molecular para su identificación y entender mejor su comportamiento. El continuo avance en tecnología del ADN recombinante ha tenido éxito en la mejora de levaduras vínicas tratadas mediante ingeniería genética con unos propósitos determinados, sobre todo por obtener cepas capaces de llevar a cabo una fermentación rápida y eficaz, además de mejorar las características organolépticas del vino, a pesar de que el uso de levaduras genéticamente modificadas (GMOs) no está autorizado en la mayoría de los países productores de vino (Butzke y Bisson, 1996; Querol y Ramón, 1996; Pretorius, 2000; Dequin, 2001; Dequin et al., 2003).

Dado el elevado uso de las levaduras comerciales en enología desde la década de los 80, es necesario evaluar las posibles adaptaciones e impacto ecológico que éstas pueden tener como consecuencia de su liberación a través de las aguas residuales, orujos, maquinaria agrícola, etc al medio ambiente. Se han llevado a cabo estudios para evaluar la capacidad de supervivencia y colonización de levaduras comerciales en Sudáfrica, Francia y Portugal (Van der Westhuizen et al., 2000a, 2000b; Valero et al., 2005). Valero et al. (2005) encontraron que la presencia de levaduras comerciales en el viñedo tenía lugar entre 50-200 m de la zona de diseminación, encontrándose muy raramente a distancias más alejadas. No obstante es necesario profundizar en este tipo de estudios de impacto ambiental de estas levaduras comerciales, ya que pueden servir como modelo para la evaluación del impacto ambiental que podría tener la utilización de levaduras modificadas genéticamente en enología. Por otra parte no hay estudios donde se analice la presencia de estas levaduras comerciales

en otros nichos del viñedo distintos a las uvas. Por ello son necesarios estudios que profundicen en este aspecto de manera directa, como es el que se presenta en esta memoria.

## 2.5. Identificación molecular de levaduras vínicas

Hasta hace poco más de una década, la identificación y caracterización de levaduras vínicas, se ha venido realizando por técnicas de taxonomía clásica, basadas en sus características morfológicas, fisiológicas y bioquímicas (Kreger-van Rij, 1984; Kurtzman y Fell, 1998; Barnett et al., 2000). La identificación en base a las características morfológicas y fisiológicas requiere la realización de numerosas pruebas, lo cual es largo, laborioso y complejo. La inexistencia de un método estandarizado para la realización de dichas pruebas de identificación, hace que los resultados dependan de la técnica empleada, lo que conduce a identificaciones erróneas (Yarrow, 1998).

La caracterización de levaduras a nivel de especie y cepa es de relevancia desde el punto de vista industrial, debido a que muchos grupos forman parte de la microbiota natural de los alimentos y bebidas fermentadas o participan en el proceso de obtención de los mismos (Orberá, 2004). De ahí la necesidad de poseer métodos de identificación rápidos, precisos y sencillos, que puedan ser aplicados al control de calidad en la industria, con el fin de asegurar que la cepa de partida y la que dé lugar al producto final sea la misma. En enología, es de enorme importancia obtener una rápida información sobre la composición y dinámica de las poblaciones de levaduras existentes durante el proceso

de vinificación, lo cual ayudará a controlar la fermentación y consecuentemente la calidad del vino (Capece, 2002).

Para superar estos inconvenientes, se han desarrollado otros métodos de identificación basados en el estudio de las moléculas de ADN y ARN. La identificación molecular de las levaduras aisladas se ha llevado a cabo a través de modernas técnicas de biología molecular que han permitido hacer una clasificación más precisa, llegando a diferenciar cepas de una misma especie. Las técnicas moleculares más rápidas son las basadas en la técnica PCR (*Polimerase Chain Reaction*) desarrollada por Saiki et al. (1985 y 1988), una de las técnicas más utilizadas es aquella que se basa en el estudio de las secuencias de los espaciadores intergénicos: regiones ITS del ADNr (ADN ribosómico) mediante PCR, que se caracteriza por su fácil manipulación y reproducibilidad. Dlauchy et al. (1999), usaron esta metodología para amplificar el gen ribosomal 18S y la región intergénica ITS1 de 128 especies asociadas principalmente con alimentos, vino, cerveza y refrescos, también utilizada y mejorada por Redzepovic *et al*. (2002). Otra región ribosomal muy útil para diferenciar a nivel de especie es la que incluye el gen 5.8S y las regiones intergénicas adyacentes ITS1 e ITS2 que se amplifican mediante los cebadores ITS1 e ITS4, descritos por White et al. (1990). Los productos de amplificado de distinto tamaño corresponden a especies diferentes, sin embargo, cuando los amplificados son del mismo tamaño no siempre pertenecen a la misma especie y es necesario recurrir a la digestión de estos productos para llegar a la identificación definitiva mediante el análisis de los RFLP (*Random Fragments Lengh Polymorphic*). Los productos de PCR se someten a digestión con endonucleasas específicas (*HaeIII, HinfI, CfoI, DdeI*) dando lugar a los fragmentos de restricción, los cuáles nos llevarán a la correcta identificación de la especie. Esta técnica fue aplicada para la rápida identificación de levaduras vínicas por Guillamón et al. (1998),

posteriormente extendida a algo más de 191 levaduras (Esteve-Zarzoso et al., 1999; Fernández-Espinar et al., 2000, 2001; Mesa et al., 2000; de Llanos Frutos et al., 2004) relacionadas con alimentos y bebidas.

Otras técnicas basadas en PCR y muy útiles para la identificación de levaduras a nivel de cepa son RAPD-PCR (*Random Amplified Polymorphic DNA-Polymerase Chain Reaction*) desarrollada por White et al. (1990) y más recientemente la PCR-SSR (*Simple Sequence Repeats)* o de microsatélites (Field y Wills, 1998; González-Techera et al., 2001; Pérez et al., 2001; Hennequin et al., 2001; Schuller et al., 2004; Bradbury et al., 2005; Malgoire et al., 2005; Legras et al., 2005; Vaudano y García–Moruno, 2008), uno de los métodos más fiables, modernos y prometedores para la identificación interespecífica de *S. cerevisiae.*

# Capítulo 3

# INFLUENCE OF THE FARMING SYSTEM AND VINE VARIETY ON YEAST COMMUNITIES ASSOCIATED WITH GRAPE BERRIES

*International Journal of Food Microbiology 45 (2011) 132-139.*

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# Capítulo 3

# 3. Influence of the farming system and vine variety on yeast communities associated with grape berries

***"You have only to drink wines from specifically different vineyards to know that the notion of terroir is true"***

- *From Robert Geddes (2007), "A good nose and great legs: the art of wine from the vine to the table"*

El uso de cepas seleccionadas de *S. cerevisiae* ha mejorado los procesos fermentativos y la calidad de los vinos, pero su uso continuado conlleva a una colonización de las bodegas dónde son utilizadas, con la consecuente eliminación de la biodiversidad microbiana de las mismas. Esto podría conllevar a la estandarización de diferentes estilos de vino. Debido a esto el viñedo constituye, probablemente, la principal reserva de levaduras autóctonas de interés enológico, por lo que es necesario preservar e incluso favorecer la presencia de especies fermentativas en el mismo.

En este sentido, se ha diseñado un plan de muestreo en viñedos de la Comunidad de Madrid durante tres años, para evaluar la influencia de distintos parámetros agronómicos sobre la biodiversidad de levaduras fermentativas de la uva. En este capítulo se presentan los resultados obtenidos, teniendo en cuenta los siguientes parámetros: el sistema de producción agrícola y la variedad de vid. Se analizaron dos viñedos (convencional y ecológico) con tres variedades de vid diferentes (Syrah, Garnacha y Barbera). De las 27 muestras tomadas en cada uno de los viñedos, 24 procedentes del viñedo ecológico fermentaron de manera espontánea, y 12 del convencional. Se aislaron un total de 1080 colonias

de levaduras, de las cuales 874 cepas fueron no-*Saccharomyces* y 206 pertenecieron al género *Saccharomyces*. Los resultados obtenidos indican una clara influencia de los tratamientos fitosanitarios empleados en el viñedo sobre las poblaciones de levaduras asociadas a la uva. Por primera vez se emplean índices de Ecología clásica para evaluar la riqueza de especies (*S*), la biodiversidad (*H'*) y la dominancia (*D*) de las especies estudiadas, con el objetivo de estudiar la ecología de las fermentaciones espontáneas de mostos obtenidos en ambos viñedos. Además, *Candida sorbosa* y *Pichia toletana* se describen por primera vez en la "D.O. vinos de Madrid".

## Abstract

Wine production in most countries is based on the use of commercial strains leading to the colonisation of the wineries by these strains with the consequent reduction of autochthonous biodiversity. This implies that wine styles could therefore become standardised. The vineyard could be an important source of native yeasts of oenological interest. For this reason the objective of this study was to compare two agronomic conditions with the aim of preserving yeast biodiversity in the vineyard. A three year sampling plan was designed to evaluate the influence of different agronomic parameters on the biodiversity of fermentative grape yeasts. Thus two vineyards, one organic and one conventional, with three different grape varieties (Shiraz, Grenache and Barbera) were chosen. In total, 27 samples were collected from both vineyards. Of these, 1080 colonies were isolated and a total of 9 species were identified. The strains identified as *Saccharomyces cerevisiae* were genotyped by microsatellite analysis obtaining nine different electrophoretic patterns. Classical ecology indexes were used to obtain the richness (*S*), the biodiversity (*H'*) and the dominance (*D*) of the species studied. The

results indicated a clear influence on grape associated yeast diversity of the phytosanitary treatment used in the vineyard. This is the first time that classical ecology indexes have been used to study the ecology of the spontaneous fermentation of grape musts and the species *Candida sorbosa* and *Pichia toletana* have been described in vineyards of the Madrid winegrowing region.

## 3.1. Introduction

Grapes are a primary source of natural yeasts in wine production. The composition and properties of different grape varieties have been extensively investigated (Bauza et al. 2007; Chavan et al. 2009; Clemente-Jimenez et al. 2005; Francesca et al. 2010; Pérez-Lamela et al. 2007; Raspor et al. 2006;). Thus, wine quality is influenced, partially, by the composition of the grape juice and by the microorganisms present in the fermentation process (Callejón et al. 2010). Species of the genera *Candida*, *Hanseniaspora*, *Hansenula*, *Issatchenkia*, *Kluyveromyces*, *Metschnikowia*, *Pichia*, *Saccharomyces*, *Torulaspora* and *Zygo-saccharomyces* are known to be present on the surface of grape berries (Chavan et al. 2009; Francesca et al. 2010; González et al. 2007; Li et al. 2010; Renouf et al. 2005). The population density and diversity of indigenous yeasts on grape berries are intricately linked to numerous factors such as the climatic conditions, the geographical location of the vineyard, the ripeness of the grape berries, the age of the vineyard, the soil type, the grape variety, the application of antifungals and the technique used to harvest (Chavan et al. 2009; Combina et al. 2005; Fleet et al. 2002; Li et al. 2010; Nisiotou and Nychas, 2007; Pretorius, 2000; Raspor et al. 2006; Valero et al. 2007; Valero et al. 2005). However, insufficient quantitative data is available to establish general conclusions on the influence of these factors. Winemakers recognise that

some indigenous yeast species may enhance or damage the quality of wine, a deeper knowledge of the effects of these factors on the yeast ecology of grapes is required.

Moreover, relevant questions concerning the microbial ecology of traditional winemaking need an answer: Is the development of a fermented must driven by the process or by the substratum? Can different yeast communities yield similar final products? What is the biodiversity of the microbiota involved in spontaneous fermentations? A better knowledge of the ecology of grape yeast communities would have direct consequences for winemaking. Molecular biology techniques can be used to identify and to compare whole yeast microbiota of spontaneously fermented musts from different grape berries. Traditional biodiversity metrics rely on species counts and/or some composite indexes, like Shannon-Wiener's or Simpson's. Ecological indexes are widely used in classical ecology but until recently they were not applied to microbial ecology and food microbiology (Ampe and Miambi, 2000; Hill et al. 2003), and so far they have not been applied to wine microbiology. These indexes have the benefit of being relatively easy to understand and easy to calculate, having a long history of application. Richness and diversity are important ecological state variables, but retain only a small proportion of the available information that describes the concept of biodiversity (Lamb et al. 2009).

Wheeler and Crisp (2009) suggest that organic grapes provide higher quality to the final wine than the conventional grapes for red but not white varieties. Shiraz, Grenache and Barbera (red varieties) are known to be easy-growing grapevines, as well as being resistant to the most common vineyard diseases. In the Madrid region (Spain) these varieties have a special interest, due to their easy adaptation to the soil, climatic conditions and other parameters which characterise this area.

Furthermore, these grapevines are used to obtain some of the wines produced in the wineries of the “D.O. Vinos de Madrid”.

The use of selected strains of *S. cerevisiae* has improved the fermentative processes and the quality of wines, but their continual use has led to a colonization and consequent elimination of the native microorganisms present in the wineries. Hence, because the vineyard may be the main reservoir of native yeasts of oenological interest, it is necessary to preserve, and even encourage, the presence of fermentative species in it. In fact, the use of native yeast strains is preferable since they are better acclimatised to the environmental conditions and assure the maintenance of the typical sensory properties of the wines of a given region (Callejón et al. 2010).

Consumers’ concern about the quality of food and beverages, particularly regarding how, when and where these products are produced is on the increase. The effect of the farming system on the environment is also a cause of concern. Organic food is likely to contain lower residues of agricultural chemicals than nonorganic food. The extensive literature on the food quality differences between organic and conventional production systems provide some overall evidence that the organic system gives higher quality (Baker et al. 2002; Benbrook, 2005; Tarozzi et al. 2006; Woese et al. 1997).

The aim of this study was to compare the yeast population density and diversity in grape juice fermentations using three grape varieties harvested from vineyards managed by conventional and organic viticultural practices in order to obtain precise information about the influence of these two parameters on the composition and evolution of yeast communities associated with grape berries.

# 3.2. Materials and Methods

## *3.2.1. Sampling plan and fermentation procedure*

This study was performed from 2006 to 2008 in two vineyards (organic and conventional) with Shiraz, Grenache and Barbera (*Vitis vinifera* L) grapevine varieties located in the Madrid winegrowing region, Spain (40° 8' 1.5864" N, -3° 22' 26.9754" W, 743 m altitude). The distance between both vineyards was 398 m. The climatological data was taken from the vineyard weather station. For these three years the data is as follows: for 2006, 2007 and 2008, the mean air temperatures during July, August and September was 23.5 ºC, 23.2 ºC and 23.2 ºC, respectively. Regarding the mean of precipitations for these three months, the data obtained was 8.3 mm, 6.6 mm, and 27.8 mm. The grapes were harvested in both the conventional and organic vineyards, both with vertical trellises facing in the direction of the gradient with Guyot pruning and bare soil by tillage. The irrigation was performed through a drip system, placing a drip every 75 cm and with a water flow of 2.2 L/hour, resulting in 150 hours/year. The conventional vineyard was treated with agricultural chemicals, such as Glyphosate 37% (Roundup plus Monsanto) at the rate of 6 L/Ha as herbicide, Neoron 50 (Bromopropylate 50% p/v, EC. Syngenta) was applied as miticide at the rate of 150 cc/HL, Kelteran 6/16 (Tetradifon 6% and Dicofol 16% w/v, EC. Aragonesas) were applied as insecticide and nematicide at the rate of 200 cc/HL and TOPAS 10 (Penconazole 10% p/v, EC. Syngenta) was applied as fungicide. All products were applied either around or directly onto the grape vines. The organic vineyard was treated only with P-300/100 micronized sulfur (98.5% sulfur, DP, Afepasa) at the rate of 20-30 kg/Ha.

In order to evaluate the diversity of yeast communities during the last stage of grape ripeness and harvest, three sampling campaigns were performed. This study was carried out over a period of 3 consecutive years (2006, 2007 and 2008). Bunches sampled were always collected from the same plant, facing in the same direction. With the present experimental design, 18 grape samples were collected every year, 9 grape samples for every farming system (3 samples for every different variety). Approximately 2 kg of grapes, stems included, were harvested in aseptic conditions from each sampling point and placed directly into sterile bags, which were transported to the laboratory in portable refrigerators with plastic ice blocks and processed within 2 hours.

At the laboratory, grapes were squeezed by hand in the plastic bags, opened in the laminar airflow bench, and 80 mL of juice was poured into 100 mL sterile fermenters. The fermenters are flasks with two openings, one at the top for filling and the other in the middle for sampling. The two openings were taped with a rubber stopper, the top one with a capillary to allow the $CO_2$ of the fermentation to escape. About 50 mL of the must were centrifuged for 5 minutes at 5000 rpm, the supernatant was used to measure its pH with a pH-meter (Crison GLP21, Barcelona, Spain) and Brix degree by refractometry (Atago digital refractometer model CO., LTD. Tokyo, Japan) .

The fermenters with 80 mL of must were placed in a temperature controlled chamber at 20 ºC with mechanical agitation (150 rpm). The fermentation progress was monitored daily by weight loss determination.

### *3.2.2. Yeast isolation*

The yeast community present in the fermentation was evaluated when the weight of the must was reduced by 70 g/L, corresponding to the

consumption of about two thirds of the sugar content. Ten-fold dilutions of must were spread on plates with YPD medium (yeast extract 1% w/v, meat peptone 1% w/v, glucose 2% w/v and agar 2% w/v) and incubated for 24 – 48 hours. Microbial density was expressed as colony forming units per milliliter (cfu/mL). Thus, 30 colonies were randomly selected from each spontaneous fermentation.

### *3.2.3. DNA extraction and quantification from isolates*

DNA extraction from yeast isolates was carried out using a commercial kit (ArchivePure DNA Purification System, 5 Prime, Germany), following the instructions provided by the manufacturer, but centrifuging at 14.000 rpm. The DNA was then stored at -20 ºC.

An UV-Vis spectrophotometer (Nanodrop 1000, Thermo Fisher Scientific Inc.) was used to calculate the quantity of DNA extracted, covering a spectral range from 220 to 750 nm.

### *3.2.4. Molecular identification of isolates*

- *PCR-RFLP analysis*

The Internal Transcribed Spacers (ITS1 and ITS2) of 5.8 S rDNA gene regions were amplified using the primers ITS1 and ITS4 primers (Sabaté et al. 2002; White et al. 1990). Hence, 1.5 µL of the DNA previously extracted from each isolate strain was resuspended in 18.5 µL of PCR mixture containing 0.4 µL of ITS1 and ITS4 (MWG Biotech AG , Ebersberg, Germany), 0.4 µL of dNTP (0.2 mM , Promega, Madison, WI, USA), 1.6 µL of $MgCl_2$ (2 mM , 5 Prime, Germany), 2 µL of Buffer 1X $NH^{4+}$ ( 5 Prime, Germany), 14.5 µL of pure water and 0.2 µL of Taq-polymerase (0.05 U/µL, 5 Prime, Germany). The rDNA was amplified in a

thermocycler (Primus 96, Peqlab. USA), following a cycle started by an initial denaturing at 95 ºC for 5 minutes; 35 cycles of denaturing at 95 ºC for 1 minute; annealing at 55 ºC for 1 minute; extension at 72 ºC for 1.5 minutes; and a final extension step of 7 minutes at 72 ºC. The resulting PCR product was stored at 4 ºC. Five micro litres of the resulting PCR product were digested according to the manufacturer's instructions with three restriction enzymes: *CfoI*, *HaeIII* and *HinfI* (Promega, Madison, WI, USA).

Both PCR products and their restriction fragments were run on a 1.4% (w/v) and 2.5% (w/v) agarose gel (Pronadisa, Labs Conda, Spain) in 1X Tris-Borate EDTA buffer (Sigma-Aldrich, USA) at 100 and 150V, respectively for 90 minutes. The gel was stained with ethidium bromide (5 µl/mL, Applichem, USA). DNA fragment sizes were determined by comparison with a molecular marker 100 bp ladder (Promega, Madison, WI, USA). To visualise the bands, a U.V. light (Spectroline U.V. transilluminator), was used, as well as a camera (Gel Logic 200 Imaging System, Kodak, NY, USA) to scan the data. The results were then processed using Molecular Kodak Image Software.

Yeasts were indentified to species level by comparing the amplified product and their restriction fragment sizes with the sizes described elsewhere (Esteve-Zarzoso et al. 1999; Fernández-Espinar et al. 2000; Guillamón et al. 1998), and with the profiles included in the data base of the Spanish Type Culture Collection (CECT). Also, in each amplification and restriction case, some certified yeast strains (*Torulaspora delbrueckii* CECT1015, *Pichia guilliermondii* CECT11029, *Metschnikowia pulcherrima* CECT10071, *Pichia toletana* CECT11493, *Pichia anomala* CECT1110, *Saccharomyces cerevisiae* CECT1176, *Kluyveromyces thermotolerans* CECT1962, *Candida sorbosa* CECT11204 and *Candida stellata* CECT11918) obtained from the Spanish Type Culture Collection (CECT) were used as patterns.

- *PCR – RAPD analysis.*

The DNA extracted was diluted with milli-Q water to a concentration ranging from 20 to 80 ng/µL. For this technique, the primer OPB – 15 was used (MWG Biotech AG, Ebersberg, Germany), containing the following sequence: 5'- GGAGGGTGTT -3'. One micro litre of the diluted DNA from each isolate strain was resuspended in 19 µL of PCR- RAPD mixture containing 2 µL of OPB - 15 (MWG Biotech AG , Ebersberg, Germany), 0.8 µL of dNTP (0.4 mM Promega, Madison, WI, USA), 2 µL of $MgCl_2$ (2.5 mM, 5 Prime, Germany), 4 µL of Buffer 1X $NH^{4+}$ (5 Prime, Germany), 9.7 µL of pure water and 0.5 µL of Taq-polymerase (0.05 U/µL, 5 Prime, Germany). The rDNA was amplified in the thermocycler (Primus 96, peqlab USA) with the following programme: 4 min at 95 ºC, 45 cycles of 1 min at 95 ºC, 1 min at 36 ºC, 2 min at 72 ºC and finally, 5 min at 72 ºC.

Amplified products were separated on an agarose gel (2.5% w/v) with 5 µl/mL of ethidium bromide (Applichem, USA), using 1X TBE as buffer at 150 V for 90 minutes. Promega ladder marker set 1 Kb molecular weight marker (Promega, Madison, WI, USA) was used. The data obtained was processed as stated before.

- *Microsatellite Multiplex PCR analysis*

The PCR reaction mix and the amplification protocols followed were the same as those used by Vaudano and García-Moruno (2008). Amplified products were separated on an agarose gel (2.5% w/v) with 5 µl/mL of ethidium bromide (Applichem, USA), in 1X TBE buffer (Sigma-Aldrich, USA) at 100 V for 90 minutes. DNA fragment sizes were determined by comparison with a molecular marker (100 bp ladder, Promega, Madison, WI, USA).

The banding patterns were processed with cluster analysis software (Bionumerics, Applied Maths, Keistraat, Belgium) using a Dice binary similarity index, and the dendrogram was built with the UPGMA method. Cophenetic correlation was applied to ascertain reliable and unreliable clusters as described by Rossetti & Giraffa (2005). Moreover, fragment differentiation and allele size determination was performed by single capillary automatic electrophoresis in ABI 3130 Genetic Analyzer (Applied Biosystem).

### *3.2.5. Statistical analysis*

Classical Ecology indexes were used to obtain the richness (*S*), the biodiversity (*H'*) and the dominance (*D*) of the species studied:

- Species Richness (*S*): this is the simplest measurement of diversity, being defined as the number of the species found in a defined area.
- Shannon-Wiener index (*H'*): used to obtain the general biodiversity:

$$H' = - \Sigma^{S} p_i \log_2 (p_i)$$

Where *S* is the number of species and $p_i$ is the proportion of the sample belonging to its species.

- Simpson's index (*D*), which gives a strong weighting to the dominant species:

$$D = \Sigma^{S} (p_i)^2$$

Where *S* is the number of species and $p_i$ is the proportion of the sample belonging to its species. Since *D* is the probability that two random individual isolates belong to the same species, Simpson's index of diversity is generally calculated as the complement of *D*:

$$1 - D = 1 - \Sigma (p_i)^2$$

In order to decide whether to accept or reject the null hypothesis, variance analysis (ANOVA) was performed to test the main effects of the factors studied, following the procedure suggested by Moreno (2001), by means of the SPSS (v.16.0) for windows statistical package.

## 3.3. Results and discussion

### *3.3.1. Sampling sites and characterization of isolated yeast*

The issue of this work is the study and evaluation of the influence of two agronomic parameters, the grape variety and the farming system, on the yeasts present in the must fermentations from grape berries obtained from vineyards managed by conventional viticultural practices and organic viticultural practices.

In order to obtain more detailed data, this study was carried out over a period of 3 consecutive years (2006, 2007 and 2008). Every year, 18 grape samples were collected 9 for each farming system (54 samples in total). The musts obtained were in optimal pH and Brix degree conditions to conduct the spontaneous fermentations in presence of fermentative microorganisms (Table 3.1).

**Table 3.1.** Brix degree and pH for 18 musts obtained from different grape varieties in both organic and conventional vineyards (Mean ± S.D.).

| | Organic vineyard | | | Conventional vineyard | | |
|---|---|---|---|---|---|---|
| | Shiraz | Grenache | Barbera | Shiraz | Grenache | Barbera |
| ºBrix | 22.5 ± 1.1 | 24.5 ± 1.1 | 21.14 ± 2.4 | 24.6 ± 2.1 | 23.3 ± 2.5 | 23.7 ± 1.6 |
| pH | 3.23 ± 0.1 | 3.3 ± 0.1 | 2.9 ± 0.2 | 3.2 ± 0.2 | 3.3 ± 0.2 | 3.0 ± 0.1 |

Thus, 13, 14, and 9 samples completed spontaneous fermentations in 2006, 2007 and 2008, respectively. A total of 1080 colonies were isolated when the weight of the must was reduced by 70 g/L, from these fermentations, 876 corresponding to non-*Saccharomyces* yeast and 204 to *Saccharomyces* strains. The global data of the fermentations and the distribution of the yeast strains isolated are shown in Table 3.2.

**Table 3.2.** Distribution of general data of the fermentations and yeast strains isolated by years.

| | Vintage | | | |
|---|---|---|---|---|
| | 2006 | 2007 | 2008 | Total |
| Samples | 18 | 18 | 18 | 56 |
| Spontaneous Fermentations | 13 | 14 | 9 | 36 |
| Isolates of *Saccharomyces* | 30 | 108 | 66 | 204 |
| Isolates of non-*Saccharomyces* | 360 | 312 | 204 | 876 |
| Total of isolates | 390 | 420 | 270 | 1080 |

Over the three years, a large proportion of non-*Saccharomyces* strains were found after fermentation, representing 81% of all the yeasts isolated and only 19% of the isolations turned out to be *Saccharomyces* strains. Taking into consideration the fact that fermentation was used as an enriching medium in order to favour the growth of fermentative yeasts, such as *S. cerevisiae*, a high proportion of non-*Saccharomyces* yeasts were isolated. The PCR and RFLP analysis of the isolates obtained from the fermentations show an important variation of the size of the fragments for the different species. The yeast species identified and their rDNA gene RFLP patterns obtained, from 2006 to 2008, are indicated in Table 3.3.

A wide range of yeast species were found throughout the different vineyards during the three years. 9 species were identified, corresponding to *Candida sorbosa* (anamorphic *Issatchenkia occidentalis*), *Candida stellata*, *Hanseniaspora guilliermondii, Kluyveromyces thermotolerans*, *Metschnikowia pulcherrima*, *Pichia anomala*, *Pichia toletana*,

*Saccharomyces cerevisiae* and *Torulaspora delbrueckii* (Table 3.3). The RAPD technique has the ability to identify the yeasts at species level and, occasionally, at strain level as well. As shown in Fig. 3.1, the results obtained with this technique confirm that all strains studied are included in the 9 species listed.

The presence of these species was previously described in other surveys of yeast microbiota in wine (Chavan et al. 2009; Di Maro et al. 2007; Francesca et al. 2010; Li et al. 2010; Mills et al. 2002; Pretorius, 2000; Ribéreau-Gayon et al. 2006; Romancino et al. 2008; Tofalo et al. 2009; Zott et al. 2008). In our work, *K. thermotolerans*, *S. cer*evisiae, *H. guilliermondii* and *C. stellata* were the most abundant species, representing 32.69%, 18.89%, 18.43% and 15.93% respectively. *P. anomala, T. delbrueckii and M. pulcherrima* were present in lower percentages: 6.48%, 4.35% and 2.78%. *P. toletana* and *C. sorbosa* were found only occasionally (0.37% and 0.09%) in the vineyard.

**Table 3.3.** Species identified in 2006, 2007 and 2008. Size of the PCR products and the restriction fragments of the species obtained with three different endonucleases (*HaeIII, CfoI and HinfI*).

| Species | *AP (bp) | Restriction fragments size (bp) | | | Total of isolates | % of isolates |
|---|---|---|---|---|---|---|
| | | *Hae* Ⅲ | *Cfo* Ⅰ | *Hinf* Ⅰ | | |
| *Metschnikowia pulcherrima* | 400 | 280 + 95 | 210 + 80 | 190 | 30 | 2.78 |
| *Candida stellata* | 500 | 490 | 210+115+70 | 230+230 | 172 | 15.93 |
| *Candida sorbosa* | 600 | 600 | 560 | 315 | 1 | 0.09 |
| *Pichia anomala* | 630 | 620 | 550 | 310+310 | 70 | 6.48 |
| *Kluyveromyces thermotolerans* | 700 | 300+210+85 | 305+280 | 355 | 353 | 32.69 |
| *Pichia toletana* | 700 | 600 | 625 | 375 | 4 | 0.37 |
| *Hanseniaspora guilliermondii* | 775 | 775 | 340+320+105 | 360+200+160 | 199 | 18.43 |
| *Torulaspora delbrueckii* | 800 | 750 | 320+210+140+100 | 410+375 | 47 | 4.35 |
| *Saccharomyces cerevisiae* | 850 | 325+250+185+150 | 375+325+150 | 375+365+110 | 204 | 18.89 |

**AP= 5.8S-ITS amplified product size*

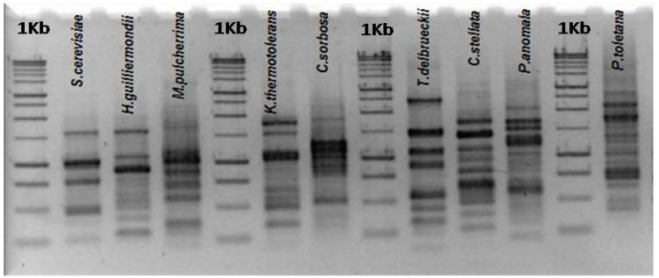


**Fig 3.1.** Identified yeast profiles obtained by RAPD-PCR.

The 204 strains identified as *S. cerevisiae* were genotyped by microsatellite multiplex PCR analysis (Vaudano and García-Moruno, 2008) using SC8132X, YOR267C and SCPTSY7 primers. Nine different electrophoretic patterns named as A, B, C, D, E, F, G, H and I were found. The allele size obtained by single capillary automatic electrophoresis and their frequency are indicated in Table 3.4.

**Table 3.4.** Microsatellite patterns and the frequency of *Saccharomyces cerevisiae* strains isolated in the three years.

| Genotype | Strains of *S. cerevisiae* | Allele size (bp) | | | | | |
|---|---|---|---|---|---|---|---|
| | | SCPTSY7-1 | SCPTSY7-2 | SC8132X-1 | SC8132X-2 | YOR267C-1 | YOR267C-2 |
| A | 55 | 292 | 292 | 212 | 310 | 308 | 389 |
| B | 35 | 269 | 269 | 193 | 193 | 421 | 421 |
| C | 31 | 261 | 312 | 155 | 212 | 389 | 389 |
| D | 18 | 271 | 271 | 206 | 206 | 389 | 389 |
| E | 21 | 280 | 280 | 209 | 209 | 389 | 389 |
| F | 28 | 280 | 280 | 209 | 209 | 407 | 407 |
| G | 14 | 261 | 261 | 212 | 212 | 389 | 389 |
| H | 1 | 261 | 269 | 193 | 212 | 389 | 421 |
| I | 1 | 286 | 286 | 181 | 181 | 389 | 389 |

## *3.3.2. Agricultural practices (conventional and organic)*

A total of 9 samples were collected from each vine variety (Shiraz, Grenache and Barbera) in both farming systems (organic and conventional), 3 for each year (2006, 2007 and 2008). For the organic system, 8 and 7 samples completed spontaneous fermentations in Shiraz and Grenache, respectively, while in Barbera it occurred in all of the samples. For the conventional vineyard, 4, 3 and 5 musts completed the spontaneous fermentation for Shiraz, Grenache and Barbera varieties respectively. A total of 876 non-*Saccharomyces* were isolated, 541 (62%) for the organic and 335 (38%) for the conventional vineyard. On the other hand, 204 *Saccharomyces* were found, 178 (87%) for the organic and 26 (13%) for the conventional vineyard (Table 3.5).

**Table 3.5.** Distribution of the global data obtained by farming system (organic and conventional vineyard) and variety (Shiraz, Grenache and Barbera) in the three years studied.

| Biological defence system | Organic Vineyard | | | Conventional Vineyard | | |
|---|---|---|---|---|---|---|
| Vine variety | Shiraz | Grenache | Barbera | Shiraz | Grenache | Barbera |
| Samples | 9 | 9 | 9 | 9 | 9 | 9 |
| Spontaneous fermentations | 8 | 7 | 9 | 4 | 3 | 5 |
| Isolates of *Saccharomyces* | 30 | 36 | 112 | 26 | 0 | 0 |
| Total of *Saccharomyces* | | 178 | | | 26 | |
| Isolates of non-*Saccharomyces* | 209 | 174 | 158 | 95 | 90 | 150 |
| Total of non-*Saccharomyces* | | 541 | | | 335 | |
| Total of isolates | | | 1080 | | | |

*3.3.2.1. Organic vineyard*

A great diversity of yeast species was observed in the musts obtained from the grapes harvested from the organic vineyard. In Shiraz musts, *K. thermotolerans* was the most abundant, comprising half of the isolated yeast (50.21%), followed by *S. cerevisiae* (12.55%), *C. stellata, M. pulcherrima* and *H. guilliermondii* as shown in Table 5. In Grenache musts, five species were found: *H. guilliermondii* being the majority species, followed by *K. thermotolerans, P. anomala, S. cerevisiae* and *C. stellata.* In Barbera musts, the number of *S. cerevisiae* strains was very high (41.48%). Regarding the non-*Saccharomyces* strains isolated, *H. guilliermondii* was the most abundant species followed by *K. thermotolerans*, *C. stellata* and a lower percentage of *T. delbrueckii* (Table 3.6).

With respect to fermentative yeasts, all genotypes were found within the *Saccharomyces cerevisiae* analyzed in the organic vineyard. This demonstrates the great diversity of fermentative strains.

**Table 3.6.** Distribution of the yeast species (number and percentage) isolated from musts obtained from different grape varieties harvested from the organic and conventional vineyards during the three years studied.

| Species | Organic vineyard | | | | | | Conventional vineyard | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Shiraz | | Grenache | | Barbera | | Shiraz | | Grenache | | Barbera | |
| | Isolates | (%) | Isolates | (%) | Isolates | (%) | Isolates | (%) | Isolates | (%) | Isolates | (%) |
| *S.cerevisiae* | 30 | 12.55 | 36 | 17.14 | 112 | 41.48 | 26 | 21.49 | 0 | 0 | 0 | 0 |
| *C.sorbosa* | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0.83 | 0 | 0 | 0 | 0 |
| *C.stellata* | 30 | 12.55 | 32 | 15.24 | 30 | 11.11 | 0 | 0 | 0 | 0 | 80 | 53.33 |
| *H.guilliermondii* | 29 | 12.13 | 60 | 28.57 | 80 | 29.63 | 0 | 0 | 30 | 33.33 | 0 | 0 |
| *K. thermotolerans* | 120 | 50.21 | 42 | 20 | 42 | 15.56 | 59 | 48.76 | 60 | 66.67 | 30 | 20 |
| *M.pulcherrima* | 30 | 12.55 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *P.anomala* | 0 | 0 | 40 | 19.05 | 0 | 0 | 30 | 24.79 | 0 | 0 | 0 | 0 |
| *P.toletana* | 0 | 0 | 0 | 0 | 0 | 0 | 4 | 3.31 | 0 | 0 | 0 | 0 |
| *T. delbrueckii* | 0 | 0 | 0 | 0 | 6 | 2.22 | 1 | 0.83 | 0 | 0 | 40 | 26.67 |

*3.3.2.2. Conventional vineyard*

A predominance of non-*Saccharomyces* strains were found in the isolates. *K. thermotolerans,* followed by *P. anomala* and, to a lesser extent, *P. toletana, C. sorbosa* and *T. delbrueckii* (both 0.83%) were the species isolated in Shiraz musts (Table 3.6). In musts from the Grenache variety, only two species, *K. thermotolerans* as the majority species (66.67%) and *H. guilliermondii*, (33.33%) were isolated. In the Barbera musts the isolated strains were *C. stellata* in the highest proportion, followed by *T. delbrueckii* and *K. thermotolerans.* With respect to the *S. cerevisiae* strains, they were poorly represented, being found only in the Shiraz musts (21.49%) (Table 3.6). As concerns the strain diversity of *S. cerevisiae*, a unique genotype (A) was found within the 26 isolates of this species analysed.

*K. thermotolerans* was the predominant non-*Saccharomyces* species found in the musts obtained from both vineyards. This yeast was previously found in several wine-producing regions (Kapsopoulou et al. 2007; Nurgel et al. 2005; Torija et al. 2001). It is common in hot and dry areas, such as the Madrid region. It is characterised by the high production of L-lactic acid, the low production of volatile acidity, moderate alcohol productivity and the absence of off-flavour production (Ribèreau-Gayon et al. 2006). It has also been reported that this yeast shows a reduced vitality in mixed cultures with *S. cerevisiae* (Kapsopoulou et al. 2007). *H. guilliermondii* was the second most frequent species of non-*Saccharomyces* found. Apiculate yeasts, like *H. guilliermondii*, are known to be high producers of esters and glycerol (Viana et al. 2008). Moreover, they have the ability to secrete some important enzymes, such as β-glucosidases and proteases, which interact with some precursor compounds (glycosidically bound terpenes) derived from the grapes. This

contributes to the subsequent expression of the varietal aroma, and, thereby, the wine-making process (Arroyo et al. 2010; Jolly et al. 2006; Li et al. 2010; Zott et al. 2008).

In our study, *C. stellata*, *T. delbrueckii* and *P. anomala* were also found in the vineyard with both farming systems. *C. stellata* is frequently associated with over-ripened and botrytized grapes and is able to survive in the fermenting must until the completion of the wine-making process (Csoma and Sipiczki, 2008). With respect to *T. delbrueckii*, it was more commonly isolated in the musts obtained from the grapes harvested from the conventional vineyard. This species has a strong fermentative capacity, being alcohol-tolerant (up to 10% v/v) and resistant to antiseptics. This yeast is commonly found in spontaneous fermenting musts, independently of the presence or absence of $SO_2$ (Renault, 2009). *P. anomala* is able to use a broad range of nitrogen and phosphor sources of different organic residues generated from some agricultural techniques (Passoth et al. 2006).

The proportion of other species was relatively low. *M. pulcherrima* was found only in the Shiraz musts obtained from the organic vineyard grapes. It is characterised for being a significantly proteolytic yeast, as well as being able to provide some important amino acids for the growth of *S. cerevisiae* by mid-fermentation (Fleet, 2003; Ganga and Martínez, 2004) and for displaying *β*-glucosidase activity (Fernández et al. 2000). A very low percentage (<5%) of *C. sorbosa* and *P. toletana* was isolated from the Shiraz musts obtained from the conventional vineyard. To our knowledge, this is the first time that *C. sorbosa* and *P. toletana* were described in vineyards of the Madrid region.

Biodiversity does not depend only on the number of species found (richness), but also on the relative dominance and the abundance of each. The hierarchies of the species are distributed according to the abundance, from those which are very abundant to those which are rarely found. Hence, the higher the degree of dominance of some species and the higher the rarity of others, the lower the biodiversity is.

Studies tend to report that, compared with conventional farming, the organic system is able to achieve a higher and richer abundance of species across a number of different areas. It has been shown that organic farming has higher microbial activity, biomass and biodiversity (Hole et al. 2005). Thus, depending on the agronomic practices (conventional and organic) there are differences in the distribution of the population of yeasts. An important amount of non-*Saccharomyces* yeasts and *Saccharomyces* strains were isolated in musts from the organic vineyard in comparison with the isolates from the conventional one. Regarding the yeasts' richness (*S*), seven different species were isolated in the conventional vineyard, while five species were found in the organic one. But if one takes into account the abundance of *S. cerevisiae* genotypes identified, richness (*S*) was higher in the organic vineyard. The general index of biodiversity (*H'*) and the concentration of dominance (*D*) were calculated on the basis of the number of identified species. The organic vineyard seemed to have the highest biodiversity of yeasts ($H_1$'=1.64), and the lowest concentration of dominance ($D_1$=0.22). Conversely, the conventional vineyard exhibited the lowest Shannon's index ($H_2$'=1.62) and the highest dominance index ($D_2$=0.25). Although, the *t-Student* applied to the Shannon-Wiener indexes showed that the differences were not significant.

Nevertheless, the Shannon's index and the concentration of dominance (*D*) were calculated for fermentative yeasts on the basis of the number of different genotypes found, the results were the highest biodiversity of strains of *S. cerevisiae* yeasts ($H_1$'= 1.95), and the lowest concentration of dominance ($D_1$= 0.15) on the organic vineyard. On the contrary, the conventional vineyard exhibited the lowest Shannon's index ($H_2$'= 0) and the highest dominance index ($D_2$= 1). The *t-Student* test applied to the Shannon-Wiener indexes showed that the differences are significant with $p< 0.05$.

The Shannon's index (*H*') is shown as a useful general diversity index that is influenced by both richness and evenness. It is more sensitive to changes in the abundance of those defined as "rare groups", such as *C. sorbosa* and *P. toletana*. Simpson's index (*D*) is strongly weighted by the dominant species. It may depend primarily on the ecological relevance of changes in the abundance of dominant species.

In this study, the results showed that the phytosanitary treatment affected the grape microbiota negatively reducing the abundance of yeasts isolated and the diversity, especially of fermentative yeast strains of *S. cerevisiae*. This last aspect is very interesting, given that phytosanitary treatments affect not only the grape richness of species in general but also have a specific effect on fermentative strains. The data shows a drastic reduction in the quantity and diversity of the *S. cerevisiae* strains isolated (178 vs 26 isolated and 9 vs 1 genotype, in organic and conventional vineyards, respectively). The reduction of non fermentative microbiota was less (541 vs 335 isolated and 6 vs 7 species of non-*Saccharomyces*, in organic and conventional vineyards respectively). This result is particularly relevant for winemakers, given that a larger proportion of fermentative yeasts favours the development of alcoholic

fermentation, principally for those winemakers who produce wines with spontaneous fermentations, particularly those classified as organic or natural wines.

### *3.3.3. Grape Variety*

The amount and distribution of the 1080 yeast strains identified in this study differed among the vine varieties. Nine different yeast species were found in the Shiraz grape variety, while five were isolated for both Grenache and Barbera, in the global data of two vineyards (Table 3.7). *K. thermotolerans* was the predominant species isolated for the Shiraz and Grenache musts, representing 49.58% and 34%, respectively. It was the third most abundant (17.14%) in Barbera must. *C. stellata* and a notable percentage of *H. guilliermondii* were found in all the varieties studied. *P. anomala* was isolated from Shiraz (8.31%) and Grenache (13.33%), but not in the Barbera must. *T. delbrueckii* was detected principally in Barbera musts, only one isolate was found in Shiraz must and none in Grenache must. A low percentage (<10%) of the species *C. sorbosa*, *M. pulcherrima* and *P. toletana* was found only in the musts obtained from Shiraz grapes (Table 3.7).

**Table 3.7.** Distribution of yeast species (number and percentage) isolated from musts obtained from three different grapes varieties during the three years studied.

| Species | Grape variety | | | | | |
|---|---|---|---|---|---|---|
| | Shiraz | | Grenache | | Barbera | |
| | Isolates | (%) | Isolates | (%) | Isolates | (%) |
| *S. cerevisiae* | 57 | 15.79 | 36 | 12 | 112 | 26.67 |
| *C.sorbosa* | 1 | 0.28 | 0 | 0 | 0 | 0 |
| *C.stellata* | 30 | 8.31 | 32 | 10.67 | 110 | 26.19 |
| *H.guilliermondii* | 29 | 8.03 | 90 | 30 | 80 | 19.05 |
| *K. thermotolerans* | 179 | 49.58 | 102 | 34 | 72 | 17.14 |
| *M. pulcherrima* | 30 | 8.31 | 0 | 0 | 0 | 0 |
| *P.anomala* | 30 | 8.31 | 40 | 13.33 | 0 | 0 |
| *P.toletana* | 4 | 1.11 | 0 | 0 | 0 | 0 |
| *T.delbrueckii* | 1 | 0.28 | 0 | 0 | 46 | 10.95 |

Regarding *S. cerevisiae* strains, Mortimer and Polsinelli (1999) discussed the low occurrence of *S. cerevisiae* in grapes and musts. On the other hand, Nurgel et al. (2005) reported the high presence of this fermentative species in musts obtained from white and black grapes grown in Anatolia, where there was an excessive use of sulfite in the vineyard. Our results showed the presence of *S. cerevisiae* species in all of the different musts studied: Shiraz (15.79%), Grenache (12%) and Barbera (26.67%) (Table 3.7).

With respect to the different genotypes of *S. cerevisiae* identified by microsatellite multiplex PCR, Barbera and Grenache grapevines were the most important reservoirs of fermentative yeast strains of *S. cerevisiae*, both in quantity and diversity. Five different genotypes (A, B, C, D, F) were found in Barbera musts and four in Grenache (E, G, H, I). It is important to note that all genotypes were different in both varieties. In the Shiraz grapevine variety only two genotypes were identified, the genotype C in the organic vineyard and the genotype A in the conventional one (Table 3.8), these genotypes were not exclusives to the Shiraz variety because they were also isolated in must from the Barbera variety.

**Table 3.8.** Distribution of different genotypes of *S. cerevisiae* throughout the different grape varieties fron the two vineyard: organic (OV) and conventional (CV).

| Genotype | OV | | | CV | | |
|---|---|---|---|---|---|---|
| | Shiraz | Grenache | Barbera | Shiraz | Grenache | Barbera |
| A | - | - | 30 | 25 | - | - |
| B | - | - | 35 | - | - | - |
| C | 30 | - | 1 | - | - | - |
| D | - | - | 18 | - | - | - |
| E | - | 21 | - | - | - | - |
| F | - | - | 28 | - | - | - |
| G | - | 14 | - | - | - | - |
| H | - | 1 | - | - | - | - |
| I | - | 1 | - | - | - | - |

The composition and the properties of the grapes are different depending on the vine variety. Varietal factors such as thickness of the grape skins can play an important role on the yeast microbiota present on grapes (Li et al. 2010). Thus, yeast populations and species may vary according to the berry development stage. As an example, Fleet (2003) reported an increased incidence of apiculate yeasts in mature grapes. Moreover, according to Renouf et al. (2005), when a large berry surface was available for the adhesion of yeasts and non agrochemical treatments were used, the microbial and yeast population was higher. These results are in agreement with those obtained in our study. Non-*Saccharomyces* yeasts were the most abundant species found in all the varieties (Shiraz, Grenache and Barbera) harvested from the organic vineyard. Therefore, the amount of *Saccharomyces* was higher in comparison with those isolated in the conventional vineyard. From the point of view of the non-*Saccharomyces* both in quantity and in the number of species isolated, Shiraz was the best variety. This could be due to the high resistance capacity of this variety to the most common vineyard diseases.

The number of isolates of *Saccharomyces* in the Barbera variety was higher compared to those found in Grenache or Shiraz. Moreover, the Barbera grapevine was found to have the largest quantity of *Saccharomyces* genotypes, followed by Grenache. This rich biodiversity found is very important for the food industry, especially for the oenological sector. Each identified genotype could influence the final quality of the wine and its properties, such as the capacity to form aroma. This capacity depends not only on the yeast species but also on the particular strain of the individual species (Torrens et al. 2008). Different strains of *Saccharomyces* can produce significant flavour differences

when fermenting the same must. This is a consequence of both the differential ability of wine yeast strains to release varietal volatile precursors and the different ability to synthesise the novo yeast-derived volatile compounds (Callejón et al. 2010; Swiegers et al. 2006; Vilanova et al. 2006; Wondra et al. 2001). Therefore, the selection of the proper yeast strain may be critical for the development of the desired wine style (Callejón et al. 2010; Molina et al. 2009). For this reason, although the majority of wine makers tend to use selected yeast cultures as starters, the use of autochthonous yeast strains is preferable since they are better acclimatised to the environmental conditions and assure the maintenance of the typical sensory properties of the wines of a given region (Callejón et al. 2010).

## 3.4. Conclusions

This study shows that the grape variety and the farming system have a strong influence on the yeast microbiota associated with the vineyard. The results showed that the phytosanitary treatments affected the grape microbiota negatively, reducing the number of yeasts isolated and their diversity, principally of fermentative yeast strains. Thus, the organic vineyard is presented as the best option for being a great and natural reservoir of fermentative yeasts of interest. Non-*Saccharomyces* yeast strains were the most abundant in both vineyard managements and in all the different varieties studied (Shiraz, Grenache and Barbera). Nevertheless, the organic vineyard was found to be a more important reservoir of quantity and diversity of *Saccharomyces* strains than the conventional one. The Barbera grapevine was the best variety in terms of the abundance and diversity of *Saccharomyces* yeast strains.

This is the first time that classical Ecology indexes have been used to study the diversity of the yeast populations in spontaneous fermentation of grape musts. Finally, to our knowledge, this is the first time that *C. sorbosa* and *P. toletana* have been described in vineyards of the Madrid winegrowing region. Our results strongly suggest that further analysis on the outcomes of organic viticulture in Spain will be needed to fully assess whether these farming systems preserve the biodiversity of associated microbiota. This fact is of fundamental importance in processes such as wine production, in which the associated microbiota has a special relevance.

# Capítulo 4

# INFLUENCE OF DIFFERENT FLOOR MANAGEMENT STRATEGIES OF THE VINEYARD ON THE NATURAL YEAST POPULATION ASSOCIATED WITH GRAPE BERRIES

*International Journal of Food Microbiology 148 (2011) 23-29*

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# Capítulo 4

# 4. Influence of different floor management strategies of the vineyard on the natural yeast population associated with grape berries

***"The more we learn about what's happening in soil, the more we learn about life"***

- *Quote from an elderly Japanese farmer in David Suzuki's Earth Time (1998).*

Algunas prácticas enológicas, como la masiva utilización de levaduras comerciales y como consecuencia, la colonización de las bodegas por parte de las mismas, puede contribuir a una reducción de la biodiversidad de levaduras nativas. En este sentido, el viñedo, una vez más, vuelve a ser el protagonista, ya que éste constituye un potencial reservorio de levaduras autóctonas de interés biotecnológico, como se demostró en el capítulo anterior. De este modo, sigue siendo necesaria la evaluación de la influencia de los parámetros agronómicos sobre las poblaciones de levaduras asociadas a la uva. Este capítulo muestra los resultados obtenidos en el estudio de la influencia de las diferentes estrategias de manejo del suelo del viñedo sobre la biodiversidad de levaduras presentes en la uva. Con este objetivo, se llevó a cabo un plan de muestreo en viñedos de la variedad Syrah de la "D.O. vinos de Madrid", durante los años 2006, 2007 y 2008, en tres sistemas de manejo del suelo diferentes: suelo desnudo por laboreo, suelo desnudo con herbicidas y suelo con cubierta vegetal. Los resultados muestran que mantener un viñedo con el suelo desnudo por laboreo se presenta como la mejor opción en cuanto a biodiversidad general de levaduras y la consecuente reducción en el uso de agroquímicos. No obstante, de acuerdo a los resultados obtenidos, el mantenimiento del suelo desnudo por herbicidas, en este caso glifosato, tuvo un impacto mínimo sobre la

diversidad de levaduras asociadas al viñedo, incluso se observó un ligero incremento de las poblaciones de levaduras, es decir, desde el punto de vista de las cepas con capacidad fermentativa, como es el caso de *S. cerevisiae*, en áreas donde el viñedo está sometido a problemas de erosión del suelo y las temperaturas son elevadas, una alternativa adecuada al laboreo, desde el punto de vista de la biodiversidad de levaduras, sería el uso de glifosato con el fin de mantener el suelo desnudo y evitar la degradación del mismo.

## Abstract

Some oenological practices, such as the massive utilisation of commercial yeast and the consequent colonisation of wineries, can contribute to reducing the native yeast biodiversity. In this context, the vineyard could be a reservoir of autochthonous yeasts of oenological interest. Thus, the evaluation of the influence of different agricultural parameters on the biodiversity of yeast population in the vineyard is necessary. This work shows the results of the influence of some floor management strategies of the vineyard in the natural yeast population associated with the grape-berries. With this objective, a three year sampling plan was designed in the Shiraz vineyards of the Madrid region using three floor management strategies: bare soil by tillage, bare soil maintained with herbicides and soil maintained with cover crop. The results of this study have shown that bare soil by tillage could be a sustainable recommendation for managing the soil, due to the reduced use of agrochemicals and the resulting high yeasts biodiversity. Nevertheless, the presence of herbicides in the vineyard has a minor impact on the diversity of grape associated yeast communities, and this could have increased the yeast populations. Hence, from the fermentative yeasts' (like *Saccharomyces*) point of view, in hot and arid

environments where soils may be affected by the tillage management, an alternative option could be the maintenance of the bare soil with the use of herbicides.

## 4.1. Introduction

*Saccharomyces* strains are the main focus of interest for winemakers because they are the main microorganisms of alcoholic fermentation. Moreover it is important to highlight that there is also an increasing interest in the industrial application of non-*Saccharomyces* yeasts in winemaking. Thus, several studies have shown their capacity to contribute positively to wine flavour (Fleet, 2003; Zott et al. 2008) or the biotechnological interest of their enzymatic activities (Arroyo et al. 2010; Fernández et al. 2000; Todaro et al. 2008). Furthermore, numerous studies have evaluated the different species present in the wine ecosystem and demonstrated the impact of grape conditions on yeast populations and other external factors such as climatic conditions, the geographical location of the vineyard, the ripeness of the grape berries, the age of the vineyard, the farming system, the grape variety and the application of agrochemicals (Cordero-Bueso et al. 2011; Fleet et al. 2002; Pretorius, 2000; Raspor et al. 2006; Valero et al. 2007; Valero et al. 2005; Zott et al. 2008). What happens in the vineyard with respect to the soil management strategies? Can these strategies have a significant effect on the natural yeast communities or on the final product?

The most common soil management used by growers is bare soil by tillage, but it should be pointed out that tillage is not a natural way of management of the vineyard soil, due to the breakdown of particles and destruction of the natural vegetation cover, leading to a higher loss of soil (Pastor et al. 2001). The use of chemical herbicides to prevent the growth

of weeds is an increasing viticultural practice to reduce the use of tillage. Glyphosate (N-phosphonomethyl glycine) is a commonly used herbicide known for its effective control against competing vegetation, its rapid inactivation in soil, and the low mammalian toxicity (Busse et al. 2001; Franz et al. 1997). According to Roslycky (1982), low concentrations of glyphosate have little effect on total populations of soil actinomycetes, bacteria and fungi, while high concentrations initially increase actinomycetes and bacterial population in soil. One of the consequences of the use of glyphosate is the change in the microbiological populations of soil, probably due to the result of the stimulation of the growth of fungi. This effect could be a consequence of direct or indirect interaction with other microorganisms (Araújo et al. 2003; Krzysko-Lupicka and Sudol, 2008).

On the other hand, the use of cover crops has been described as a sustainable alternative means of floor management with many advantages. Cover crops can increase the presence of organic matter and nutrients in the soil, due to aerial degradation and underground biomass. This improves some physical properties of the soil, like its porosity, its structure and the stability of the aggregates (Tesic et al. 2007). Also, it has been stated that cover crops increase the moisture retention capacity, and the cation exchange capacity of the soil, as well as being able to reduce the water runoff, prevent erosion and increase soil biological activity (Frye and Blevins, 1989). As regards the vineyard, some other additional benefits are that it contributes to the control of the weed population, and it is a means of controlling some species of nematodes that damage the vineyard (Aballay and Insunza, 2002). The great disadvantage of the ground cover is that it may affect competition for water with the vineyard (Marques et al. 2010; Pastor et al. 2001; Tesic et al. 2007). The effect of water and nutrients competition between sward

resulting from the reduced use of herbicides and vines has also been studied, the relationship being well established in cooler and more humid areas. Nevertheless, it is poorly understood in warm and dry areas (Tesic et al. 2007). In addition, several experimental works have shown that an inappropriate management affects the physical and chemical characteristics of the soil, affecting the productivity of the vineyard and grape composition (Marques et al. 2010; Murisier et al. 1999; Ovalle et al. 2007).

The aim of this study was to compare the yeast population density and diversity in must fermentations from grapes harvested from vineyards with the floor managed with three different strategies: bare soil by tillage, bare soil maintained with herbicides and soil maintained with cover crop, in order to obtain precise information about the influence of these agronomic practices on the composition and evolution of native yeast populations associated with grape berries.

## 4.2. Materials and Methods

### *4.2.1. Sampling plan and fermentation procedure*

This study was performed from 2006 to 2008 in an experimental vineyard with Shiraz grapevine variety (*Vitis vinifera* L) located in the Madrid winegrowing region, Spain (40° 8' 1.5864" N, -3° 22' 26.9754" W, and 743 m altitude). The climatological data was taken from the vineyard weather station. The mean air temperatures during July, August and September were 23.5 ºC, 23.2 ºC and 23.2 ºC, for 2006, 2007 and 2008 respectively. As regards the mean precipitations for these three months, the data obtained was 8.3 mm, 6.6 mm, and 27.8 mm. respectively. The grapes were harvested in a conventional vineyard on vertical trellises

facing the direction of the gradient with three different floor management strategies: bare soil by tillage (VBST), bare soil maintained with herbicides (VMH) and soil with cover crop (VCC). In the bare floor by tillage no herbicide was applied, and it was cover crop free. Glyphosate 37% (Roundup plus Monsanto) at the rate of 6 L/Ha was applied as herbicide. This product was applied around of the vines according to the manufacturer's recommendations considering the safety values and the maximum levels allowed by European legislation (Commission Directive 2006/60/EC of the European Communities). Glyphosate was sprayed at three times during the spring of 2006 and 2007 in April, May and June. In 2008, it was applied at four times (in spring) due to the weather conditions were humid. It is recommended to apply upto five hours prior to a forecasted rainfall. The cover crop was spontaneously formed by grasses. Irrigation was performed through a drip system, placing a drip every 75 cm and with a water flow of 2.2 L/hour, resulting in 150 hours/year.

Bunches sampled were always collected from the same plant, facing in the same direction. All the samples were collected when the grapes had got the industrial maturation stage, taking into account the Brix degree of the musts. These samples were in good sanitary conditions. With the present experimental design, 9 grape samples were collected every year, 3 samples for every floor management strategy. Approximately 2 kg of grapes, stems included, were harvested in aseptic conditions from each sampling point and placed directly into sterile bags, which were transported to the laboratory in portable refrigerators with plastic ice blocks and processed within 2 hours.

At the laboratory, grapes were squeezed by hand in the plastic bags, opened in the laminar airflow bench, and 80 mL of juice was poured into

100 mL sterile fermenters. The fermenters are flasks with two openings, one at the top for filling and the other in the middle for sampling. The two openings were taped with a rubber stopper, the top one with a capillary to allow the $CO_2$ of the fermentation to escape. About 50 mL of the must were centrifuged for 5 minutes at 5000 rpm, the supernatant was taken to measure its pH with a pH-meter (Crison GLP21, Barcelona, Spain) and Brix degree by refractometry (Atago digital refractometer model CO., LTD. Tokyo, Japan) .

The fermenters with 80 mL of must were placed in a temperature controlled chamber at 20 ºC with mechanical agitation of 150 rpm in order to maintain the homogeneity of the must and the particles in suspension because otherwise a must decantation and non homogeneous conditions would have resulted despite the small volumes. Fermentation progress was monitored daily by weight loss determination.

### *4.2.2. Yeast isolation*

The yeast community present in the fermentation was evaluated when the weight of the must was reduced by 70 g/L, corresponding to the consumption of about two thirds of the sugar content. Ten-fold dilutions of must were spread on plates with YPD medium (yeast extract 1% w/v, meat peptone 1% w/v, glucose 2% w/v and agar 2% w/v) and incubated for 24–48 hours. Thus, 30 yeast colonies were randomly selected from each spontaneous fermentation.

### *4.2.3. DNA extraction and quantification from isolates*

DNA extraction from yeast isolates was carried out using a commercial kit (ArchivePure DNA Purification System, 5 Prime, Germany), following the instructions provided by the manufacturer, but

centrifuging at 14.000 rpm. The DNA was then stored at -20 ºC. An UV-Vis spectrophotometer (Nanodrop 1000, Thermo Fisher Scientific Inc.) was used to calculate the quantity of DNA extracted, covering a spectral range from 220 to 750nm.

### *4.2.4. Molecular identification of isolates*

- *PCR-RFLP analysis*: the Internal Transcribed Spacers (ITS1 and ITS2) of 5.8 S rRNA gene regions were amplified using the primers ITS1 and ITS4 primers (Sabaté et al. 2002; White et al. 1990). Hence, 1.5 µL of the DNA previously extracted from each isolate strain was resuspended in 18.5 µL of PCR mixture containing 0.4 µL of ITS1 and ITS4 (MWG Biotech AG , Ebersberg, Germany), 0.4 µL of dNTP (0.2 mM , Promega, Madison, WI, USA), 1.6 µL of $MgCl_2$ (2 mM , 5 Prime, Germany), 2 µL of Buffer 1X $NH^{4+}$ ( 5 Prime, Germany), 14.5 µL of pure water and 0.2 µL of Taq-polymerase (0.05 U/µL, 5 Prime, Germany). The rDNA was amplified in a thermocycler (Primus 96, Peqlab. USA), following a cycle started by an initial denaturing at 95 ºC for 5 min; 35 cycles of denaturing at 95 ºC for 1 min; annealing at 55 ºC for 1 min; extension at 72 ºC for 1.5 min; and a final extension step of 7 min at 72 ºC. The resulting PCR product was stored at 4 ºC. Five micro liters of the resulting PCR product were digested according to manufacturer instructions with three restriction enzymes: *Cfo*I, *Hae*III and *Hinf*I (Promega, Madison, WI, USA).

Both PCR products and their restriction fragments were run on a 1.4% (w/v) and 2.5% (w/v) agarose gel (Pronadisa, Labs Conda, Spain) in 1X TBE buffer (Sigma-Aldrich, USA) at 100 and 150V, respectively for 90 min. The gel was stained with 5 µl/mL ethidium bromide (Applichem, USA). DNA fragment sizes were determined by comparison with a molecular marker 100 bp ladder (Promega, Madison, WI, USA). To visualise the bands, a U.V. light (Spectroline U.V. transilluminator), was used, as well as a camera (Gel

Logic 200 Imaging System, Kodak, NY, USA) to scan the data. The results were then processed using Molecular Kodak Image Software.

Yeasts were identified to species level by comparing the amplified product and their restriction fragment sizes with the sizes described elsewhere (Esteve-Zarzoso et al. 1999; Fernández-Espinar et al. 2000; Guillamón et al. 1998), and with the profiles included in the data base of the Spanish Type Culture Collection (CECT). Also, in each amplification and restriction case, some certified yeast strains (*Torulaspora delbrueckii* CECT1015, *Pichia guilliermondii* CECT10105, *Metschnikowia pulcherrima* CECT10071, *Pichia toletana* CECT11493, *Pichia anomala* CECT1110, *Saccharomyces cerevisiae* CECT1176, *Kluyveromyces thermotolerans* CECT1962, *Candida sorbosa* CECT11204 and *Candida apicola* CECT11167) obtained from the Spanish Type Culture Collection (CECT) were used as patterns.

- *PCR – RAPD analysis*

DNA extracted was diluted with milli-Q water to a concentration ranging from 20 to 80 ng/µL. For this technique, the primer OPB – 15 was used (MWG Biotech AG, Ebersberg, Germany), containing the following sequence: 5'- GGAGGGTGTT -3'. One micro litre of the diluted DNA from each isolate strain was resuspended in 19 µL of PCR- RAPD mixture containing 2 µL of OPB - 15 (MWG Biotech AG , Ebersberg, Germany), 0.8 µL of dNTP (0.4 mM Promega, Madison, WI, USA), 2 µL of $MgCl_2$ (2.5 mM, 5 Prime, Germany), 4 µL of Buffer 1X $NH^{4+}$ (5 Prime, Germany), 9.7 µL of pure water and 0.5 µL of Taq-polymerase (0.05 U/µL, 5 Prime, Germany). The rDNA was amplified in the thermocycler (Primus 96, peqlab USA) with the following conditions: 4 min at 95 ºC, 45 cycles of 1 min at 95 ºC, 1 min at 36 ºC, 2 min at 72 ºC and finally 5 min at 72 ºC.

Amplified products were separated on an agarose gel (2.5% w/v) with 5 µl/mL of ethidium bromide (Applichem, USA), using 1X TBE buffer at 150 V, for 90 minutes. A ladder marker set 1 Kb molecular weight (Promega, Madison, WI, USA) was used. The data obtained was processed as stated before.

- *Microsatellite Multiplex PCR analysis*

Strains identified as *S. cerevisiae* were genotyped with three microsatellite loci, SC8132X, YOR267C and SCPTSY7. They were used because of their high degree of polymorphism (Vaudano and García-Moruno, 2008). The PCR reaction mix and the amplification protocols followed were the same as those used by Vaudano and García-Moruno (2008). Amplified products were separated on an agarose gel (2.5% w/v) with 5 µl/mL of ethidium bromide (Applichem, USA), in 1X TBE buffer (Sigma-Aldrich, USA) at 100V, for 90 minutes. DNA fragment sizes were determined by comparison with a molecular ladder marker of 100 bp (Promega, Madison, WI, USA).

The banding patterns were processed with cluster analysis software (Bionumerics, Applied Maths, Keistraat, Belgium) using a Dice binary similarity index, and the dendrogram was built with the UPGMA method. Cophenetic correlation was applied to ascertain reliable and unreliable clusters as described by Rossetti and Giraffa (2005). Moreover, fragment differentiation and allele size determination was performed by single capillary automatic electrophoresis in ABI 3130 Genetic Analyzer (Applied Biosystem). Repeatability was tested by performing the amplification on three colony DNA extractions for each strain, and the amplification products were analysed by single capillary automatic electrophoresis in three different rounds.

## 4.3. Results and discussion

The aim of this work involved the assessment of the influence of different floor management strategies of the vineyard on native yeast populations associated with grape-berries. This study was carried out in the same experimental vineyard under the same climatic conditions and a control of some variables, such as the type of soil, antifungal management strategies, irrigation system, ripeness status of the grape berries, etc. in order to avoid the influence of these external factors over the different floor management strategies as object of this study. Nevertheless in open field experiments, the variability of the climatic conditions year by year, cannot be avoided.

Every year, 9 grape samples were collected, 3 for each floor management strategy studied. All grapes were in good sanitary conditions. On the basis of the Brix degree, the sugar content in each sample was the correct for reach a spontaneous fermentation. Regarding the pH, in 2006 was slightly low than in 2007 and 2008 (Table 4.1). However, several studies showed that a variation of medium pH between 3.0 and 4.0 did not significantly affect the growth rate or cell biomass of the yeasts (Charoenchai et al. 1998; Heard and Fleet, 1988; Gao and Fleet, 1988). Thus 4, 5, and 5 samples reached spontaneous fermentations in the VBST, VMH and VCC, respectively. The fermentation kinetics by measuring the initial sugar content and the residual sugar for each fermentation is shown in Table 4.1. In this study, we consider that fermentation was carried out when the residual sugar had decreased to less than 70 g/L, when the yeast community was evaluated. At this moment, a total of 420 colonies were isolated from these fermentations, 353 corresponding to non-Saccharomyces and 67 to

Saccharomyces strains. The global data of the fermentations and distribution of yeast strains isolated are shown in Table 4.2.

**Table 4.1.** Brix degree, pH and fermentation kinetics by measuring the initial sugar content and the residual sugar for each fermentation of the study during 2006, 2007 and 2008 for the different samples obtained from Shiraz grape berries from each floor management strategy (VBST= vineyard with bare soil by tillage; VMH= vineyard bare soil maintained with herbicide; VCC= floor with cover crop).

| | | ºBrix | pH | Initial sugar (g/L) | Residual sugar (g/L) | Time to dryness (days) | *S. cerevisiae* (Absence/Presence) |
|---|---|---|---|---|---|---|---|
| **vintage 2006** | **VBST** | | | | | | |
| | Sample 1 | 27.0 | 2.91 | 220 | 16 | 23 | Absence |
| | Sample 2 | 27.0 | 2.83 | 212 | 137 | 27 | Absence |
| | Sample 3 | 27.1 | 2.89 | 256 | 147 | 38 | Absence |
| | **VMH** | | | | | | |
| | Sample 4 | 22.4 | 2.86 | 220 | 112 | 33 | Absence |
| | Sample 5 | 23.4 | 3.01 | 231 | 106 | 36 | Absence |
| | Sample 6 | 25.8 | 3.07 | 259 | 28 | 21 | Absence |
| | **VCC** | | | | | | |
| | Sample 7 | 27.3 | 3.28 | 276 | 220 | 35 | Absence |
| | Sample 8 | 21.7 | 2.90 | 213 | 21 | 22 | Absence |
| | Sample 9 | 22.5 | 2.87 | 221 | 120 | 35 | Absence |
| **vintage 2007** | **VBST** | | | | | | |
| | Sample 10 | 25.2 | 3.35 | 252 | 89 | 35 | Absence |
| | Sample 11 | 25.7 | 3.44 | 258 | 49 | 20 | Absence |
| | Sample 12 | 20.3 | 3.25 | 197 | 0 | 12 | Presence |
| | **VMH** | | | | | | |
| | Sample 13 | 26.8 | 3.41 | 270 | 81 | 35 | Absence |
| | Sample 14 | 22.9 | 3.56 | 225 | 6 | 14 | Presence |
| | Sample 15 | 25.2 | 3.68 | 254 | 26 | 18 | Presence |
| | **VCC** | | | | | | |
| | Sample 16 | 25.7 | 3.58 | 258 | 145 | 35 | Absence |
| | Sample 17 | 19.6 | 3.22 | 188 | 21 | 28 | Absence |
| | Sample 18 | 20.5 | 3.24 | 198 | 41 | 27 | Absence |
| **Vintage 2008** | **VBST** | | | | | | |
| | Sample 19 | 27.0 | 3.37 | 272 | 254 | 22 | Absence |
| | Sample 20 | 27.0 | 3.39 | 272 | 163 | 26 | Absence |
| | Sample 21 | 27.1 | 3.41 | 274 | 250 | 23 | Absence |
| | **VMH** | | | | | | |
| | Sample 22 | 27.4 | 3.30 | 277 | 168 | 26 | Absence |
| | Sample 23 | 27.4 | 3.30 | 277 | 43 | 12 | Presence |
| | Sample 24 | 26.0 | 3.34 | 262 | 238 | 23 | Absence |
| | **VCC** | | | | | | |
| | Sample 25 | 24.0 | 3.26 | 238 | 71 | 22 | Absence |
| | Sample 26 | 27.9 | 3.31 | 284 | 98 | 22 | Absence |
| | Sample 27 | 23.6 | 3.21 | 233 | 142 | 26 | Absence |

**Table 4.2.** Distribution of general data of the fermentations and yeast strains isolated by vineyards in the 3 years.

| | Floor management strategy | | |
|---|---|---|---|
| | Bare soil by tillage | Bare soil maintained with herbicides | Floor with cover crop |
| Samples | 9 | 9 | 9 |
| Spontaneous fermentations | 4 | 5 | 5 |
| Total of isolates | 120 | 150 | 150 |
| Isolates of *Saccharomyces* | 25 | 42 | 0 |
| Isolates of non-*Saccharomyces* | 95 | 108 | 150 |

Over the three years, a large proportion of non-*Saccharomyces* strains were found after fermentation, representing 84% of the total yeast isolated against 16% of *Saccharomyces* strains. Taking into consideration the fact that fermentation was used as an enriching medium in order to favor the growth of fermentative yeasts, such as *S. cerevisiae*, a high proportion of non-*Saccharomyces* yeast were isolated. PCR and RFLP analysis of all the isolates obtained from the fermentations showed a high variation in the size fragments for the different species. The yeast species identified and the rDNA gene RFLP patterns obtained from 2006 to 2008 are indicated in Table 4.3.

The RAPD technique was used to identify yeasts at species level and, occasionally, at strain level. The results obtained with this technique confirm the identification of nine different species listed in table 4.3. The presence of these species was described in other surveys of yeast communities in wine (Cordero-Bueso et al. 2011; Di Maro et al. 2007; Esteve-Zarzoso et al. 1998; Mills et al. 2002 Pretorius, 2000; Querol and Ramon, 1997; Ribéreau-Gayon et al. 2006 Romancino et al. 2008; Tofalo et al. 2009; Zott et al. 2008). *K. thermotolerans* and *P. anomala* and *S. cerevisiae* were the most abundant species in the vineyards with 28.81% 18.10% and 15.95% respectively. *M. pulcherrima* and *P. guilliermondii* were

present with 7.14% each, and other species such as *P. toletana* and *C. sorbosa* were found in minor percentages (0.95% and 0.24%).

All strains identified as *S. cerevisiae* were genotyped by microsatellite multiplex PCR analysis and three different electrophoretic patterns (A, B, and C) were obtained. In order to facilitate understanding of the results, they have been discussed according to the floor management strategy.

**Table 4.3.** Size of the PCR products and the restriction fragments of the species identified with three different restriction endonucleases during the 3 years.

| Species | *AP (bp) | Restriction fragments size (bp) | | | Total of isolates | % of isolates |
|---|---|---|---|---|---|---|
| | | *Hae* III | *Cfo* I | *Hinf* I | | |
| *Metschnicowia pulcherrima* | 400 | 280 + 95 | 210 + 80 | 190 | 30 | 7.1 |
| *Candida apicola* | 490 | 385+88 | 210+184 | 228+133 | 60 | 14.3 |
| *Candida sorbosa* | 600 | 600 | 560 | 315 | 1 | 2.4 |
| *Pichia anomala* | 630 | 620 | 550 | 310+310 | 76 | 18.1 |
| *Pichia guilliermondii* | 625 | 298+261 | 368+115+90 | 309+285 | 30 | 7.1 |
| *Kluyveromyces thermotolerans* | 700 | 300+210+85 | 305+280 | 355 | 121 | 28.8 |
| *Pichia toletana* | 700 | 600 | 625 | 375 | 4 | 1.0 |
| *Torulaspora delbrueckii* | 800 | 750 | 320+210+140+100 | 410+375 | 31 | 7.4 |
| *Saccharomyces cerevisiae* | 850 | 325+250+185+150 | 375+325+150 | 375+365+110 | 67 | 16.0 |

**AP= 5.8S-ITS amplified product size*

### *4.3.1. Bare soil by tillage*

A total of 9 grape samples were collected from the bare soil by tillage, 3 for each year. One and three samples reached spontaneous fermentations in 2006 and 2007, respectively, while in 2008 musts obtained from this management strategy did not ferment (Table 4.1). For each sample 30 colonies were isolated (120 yeast strains isolated in total), 95 corresponding to non-*Saccharomyces* strains and 25 to *Saccharomyces*.

In 2006, two species were isolated: *K. thermotolerans* (97%) and *C. sorbosa* (3%). In 2007, *K. thermotolerans* and *P. anomala* were the most abundant species, representing both 33%, followed by *S. cerevisiae* (28%). *P. toletana* and *T. delbrueckii* were minor species 5% and 1%, respectively. As shown above, no yeast strains were isolated in 2008 (Table 4.4).

*K. thermotolerans* was the predominant non-*Saccharomyces* species in grape musts obtained from the vineyard managed by bare soil by tillage. This yeast was found in several wine-producing regions and it is common in hot and dry areas, like is the Madrid region (Cordero-Bueso et al. 2011; Kapsopoulou et al. 2007; Nurgel et al. 2005). Regarding the source of fermentative yeast species, some previous studies (Martini, 1997; Suárez-Valles et al. 2007; Vaughan and Martini, 1995) have shown that *Saccharomyces* are associated with the winery surfaces and winemaking equipment, being uncommon species in the must, except when there are damaged fruits. Others argue that the main source is the vineyard, depending on the absence or presence of each strain of *S. cerevisiae* on each vine and grape (Pretorius, 2000; Török et al. 1996).

**Table 4.4.** Distribution of yeast species (number of isolates) during spontaneous fermentations of must from grapes harvested from vineyard with bare soil by tillage, bare soil maintained with herbicides and floor with cover crop at different sampling years.

| | Bare soil by tillage | | | Bare soil mantained with herbicides | | | Floor with cover crop | | |
|---|---|---|---|---|---|---|---|---|---|
| | 2006 | 2007 | 2008 | 2006 | 2007 | 2008 | 2006 | 2007 | 2008 |
| Samples | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 |
| Spontaneous Fermentations | 1 | 3 | 0 | 1 | 3 | 1 | 1 | 2 | 2 |
| Total isolates | 30 | 90 | - | 30 | 90 | 30 | 30 | 60 | 60 |
| *Candida apicola* | - | - | - | 30 | - | - | 30 | - | - |
| *Candida sorbosa* | 1 | - | - | - | - | - | - | - | - |
| *Pichia anomala* | - | 30 | - | - | 16 | - | - | 30 | - |
| *Kluyveromyces thermotolerans* | 29 | 30 | - | - | 32 | - | - | - | 30 |
| *Metschnikowia pulcherrima* | - | - | - | - | - | - | - | 30 | - |
| *Pichia guilliermondii* | - | - | - | - | 30 | - | - | - | - |
| *Pichia toletana* | - | 4 | - | - | - | - | - | - | - |
| *Torulaspora delbrueckii* | - | 1 | - | - | - | - | - | - | 30 |
| *Saccharomyces cerevisiae* | - | 25 | - | - | 12 | 30 | - | - | - |

The results obtained in this study were in agreement with these latest findings because in 2007, *S. cerevisiae* represented 28% of the total of isolates from the vineyard, while in 2006 and 2008 no Saccharomyces genus strains were isolated in the VBST. After the analysis by microsatellite multiplex of the species identified as *S. cerevisiae* a unique genotype (A) was found.

Interestingly, the predominant species during fermentation were very different year by year. In 2006, only one must (sample 1) completed the spontaneous fermentation (Table 4.1). The yeast population isolated from this sample was belonging to *K. thermotolerans* and *C. sorbosa* species. However, in 2007, all samples reached spontaneous fermentations (Table 4.1). In the samples 10 and 11 were isolated two species of non-*Saccharomyces, P. anomala* and *K. thermotolerans*, while in the sample 12, the prevalent yeast was *S. cerevisiae*. Curiously, in the majority of the samples which completed fermentation were isolated only species of non-*Saccharomyces*. Several authors have mentioned that indigenous yeast species, such as *Hanseniaspora guilliermondii*, *K. thermotolerans*, *C. stellata*, *C. apicola* and *T. delbrueckii*, may have better ability than *S. cerevisiae* to grow during fermentations conducted at initial sugar concentrations up to 200 g/L (Benda, 1982; Kapsopoulou et al. 2007; Lafon-Lafourcade, 1983; Tofalo et al. 2009; Valero et al. 2005, 2007). Nevertheless the time to dryness of the musts containing non-*Saccharomyces* was much longer than in the samples with *S. cerevisiae*.

In 2008, samples obtained from this vineyard management strategy did not reach spontaneous fermentations because a notable population of fungi grew in the musts poured into the fermenters after three days and under conditions of 20ºC and agitation (150 rpm). Furthermore, this year, the weather conditions were three times more humid than in 2006 and

2007. According to Longo et al. (1991), a humid and rainy climate favors fungal proliferation and may alter the equilibrium between fermentative and oxidative species. Thus, the weather conditions are very important factors and its influences onto the yeast communities associated with the grape berries.

### *4.3.2. Bare soil maintained with herbicides*

In the bare soil maintained with herbicides, from the 9 grape samples collected, 3 samples reached spontaneous fermentation in 2007 while only one fermentation was completed in both 2006 and 2008 (Table 4.1). From these fermentations, 150 yeast colonies were isolated of which 28% belonged to the *Saccharomyces* and 72% to the non-*Saccharomyces* genus (Table 4.4).

Species *C. apicola* was the unique species found in 2006. *K. thermotolerans* (36%), *P. guilliermondii* (33%) and *P. anomala* (18%) were the species isolated in 2007, while in 2008 no non-*Saccharomyces* species were isolated. *S. cerevisiae* represented 13% and 100% of the isolates of 2007 and 2008, respectively. Three genotypes (A, B and C) were found within the *Saccharomyces* analyzed.

*C. apicola* was isolated from the must (sample 6) that reached spontaneous fermentation in 2006 (Table 4.1). This yeast is present in the stretch of some hymenopterous like honey bees and wasps. Several studies of non-*Saccharomyces* yeasts isolated from musts, mention *C. apicola* (Barrajón et al. 2009; De Llanos Frutos et al. 2004; Esteve-Zarzoso et al. 1999; Tofalo et al. 2009). The ability of some *C. apicola* strains to grow at 14% (v/v) ethanol is noteworthy (Tofalo et al. 2009), thus this species was able to survive in the middle or even until the end of

the fermentation (Barrajón et al. 2009), for this reason, *C. apicola* could be a species of biotechnological interest. In the second year, three species of non-*Saccharomyces* were isolated from the three musts, *K. thermotolerans* being the most common and *P. anomala* the least abundant. Both species were present in the spontaneous fermentations of samples 14 and 15 as predominant species. *S. cerevisiae* was also present in both samples but in a lower quantity (Table 4.1). These species have been also described in the vineyard managed by tillage. *P. guilliermondii* was isolated from the sample 10 as sole species in this must. In 2008 only *Saccharomyces* strains were isolated from the must (Table 4.4).

In the VMH, the number of *S. cerevisiae* strains was higher in comparison with those isolated in the vineyard with bare soil by tillage, previously commented (Table 4.4). Consequently, the amount of spontaneous fermentations from must obtained from grape berries collected was the most abundant. Furthermore, this management strategy showed the higher number of genotypes of *S. cerevisiae*; A (sample 15) and B (sample 14) in 2007 and, C (sample 23) in 2008. Genotypes A and B were not found in 2008, while genotype C was the dominant this year. The profile A was also found in the sample 12 of the VBST in 2007.

In 2008, glyphosate was applied for four times due to the weather conditions were humid in contradistinction to the three times sprayed in 2006 and 2007. This herbicide inhibits the 5-enolpyruvylshikimic acid-3-phosphate synthase, an intermediate enzyme in the aromatic amino acid synthesis via the shikimic acid pathway (Franz et al. 1997). Most living organisms, excluding plants, lack this pathway, so they are directly unaffected by the presence of glyphosate. However, the shikimic acid

pathway is ubiquitous in microorganisms (Bentley, 1990). Reports of harmful effects to microorganisms are numerous in laboratory studies (Busse et al. 2001; Krzysko-Lupicka and Sudol, 2008; Krzysko-Lupicka and Orlik, 1997; Santos and Flores, 1995). Contrary to laboratory results, most agricultural studies have shown that glyphosate do not affect (or even encourage) the presence of soil microorganisms (Busse et al. 2001; Haney et al. 2000; Johal and Huber, 2008; Roslycky, 1982). These discrepancies between the results obtained in laboratory and field studies can be partially explained by the high concentrations of herbicide used in many of these laboratory studies, and by the herbicide chemistry itself (Busse et al. 2001; Wardle, 1995). In addition, the absorption and effects of the agrochemicals on the grapevines could vary depending of the weather conditions and nutritional factors (Čadež et al. 2010).

Our findings suggest that the rate of glyphosate used (Roundup 37% Monsanto), a value ranged within the safety intervals have little or no repercussion on grape yeast communities. Besides, the use of this herbicide could have stimulated the occurrence of yeasts populations (including fermentative strains) as they might have used this glyphosate as a nutrient, as well as an energetic substrate.

### *4.3.3. Floor with cover crop*

Nine grape samples were collected from the vineyard with floor with cover crop, of which 1, 2 and 2 samples reached spontaneous fermentations in 2006, 2007 and 2008 respectively (Table 4.1). From these fermentations 150 colonies were isolated, all corresponding to non-*Saccharomyces*. Thus, the *Saccharomyces* genus was not represented in any year (Table 4.4).

In the first year, the only isolated species was *C. apicola*, a species that also appeared in the bare soil maintained with herbicides. In the second year the isolated strains were *M. pulcherrima* (50%) and *P. anomala* (50%), meanwhile in the third year the yeasts identified were *K. thermotolerans* (50%) and *T. delbrueckii* (50%) (Table 4.4). These results are in agreement with those shown by Cordero-Bueso et al. (2011); Baleiras-Couto et al. (2005), Barrajón et al. (2009), Fernández et al. (2000), Romancino et al. (2008) and Tofalo et al. (2009), who found these species in fresh musts obtained from different places. Nevertheless, they were found at different ratios between the species.

In 2007 another different species was isolated and identified, *M. pulcherrima*. The presence of this yeast in grape berries and grape musts has been described previously in Shiraz grapevine variety in the same area (Cordero-Bueso et al. 2011). In this case, it was present in a must (sample 18) from grapes collected from a vineyard with cover crop (Table 4.1). *M. pulcherrima* contributes in several different ways to fermentation and wine composition. It is characterised for being significantly proteolytic yeast (Fleet, 2003; Ganga and Martínez, 2004) and for displaying *β*-glucosidase activity (Arroyo et al. 2010; Esteve-Zarzoso, 1998; Fernández et al. 2000). Some authors have reported *M. Pulcherrima* to be inhibitory to the growth of other yeasts, such as *S. cerevisiae* (Nguyen and Panon, 1998; Romancino et al. 2008).*Saccharomyces* yeasts were not found in spontaneous fermentations from grapes from the vineyard managed with cover crop. The presence of permanent vineyard cover crops or swards has been examined on a variety of soils and climates across Europe, including light-textured soils in dry regions. Sward-vine competition reduced vine vigor and yield in cool and dry areas of Germany so drastically that, in the 1950s, cover crops were abandoned, being considered as unsustainable (Schultz and Löhnertz, 2003).

Marques et al. (2010) observed a reduction of 50% in the yield of a vineyard beneath a grass cover in the Madrid region, next to the vineyard of our study, due to the lower degree of soil moisture. A reduction in vine vigor was observed in Bordeaux vineyards when comparing the presence of different swards with traditional soil cultivation (Coulon, 2002), although the reduced vigor was not necessarily associated with a reduced yield in Spain due to the greater exposure to sunshine. However, excessive exposure of grape to direct radiation can cause sunburn and a rapid rate of ripening (White, 2009). Furthermore, cover crops affect soil temperature, because these have a higher albedo than bare soils. The energy absorbed during the day, and hence soil warming, is less under these types of cover. At night, bare soils surfaces with no vegetative cover lose heat rapidly, and soils with cover crop remain warmer than bare soils (White, 2009). This is an important factor in decreasing frost damage to green tissues, and consequently to grape berries and the yeast populations associated to them. Cover crop has also negative effects on the available nutrients present in the soil for the plant (Tesic et al. 2007).

The results obtained in this study were according to the previous studies described. The Madrid region is a hot and dry area, where the presence of floor cover crop may increase albedo and decrease water and nutrient availability. This reduction in the vineyard soil moisture could be associated with a considerably decrease in vine vigor and yield, consequently in the biodiversity of yeast populations, affecting the *Saccharomyces* genus considerably. Additionally, due to the high humidity caused by the presence of the cover crop, the fungi population may be increased in this floor. Some of the musts obtained from the vineyard with cover crop during the three year did not reach spontaneous

fermentation. Fungi populations in the musts after three days of fermentation process were observed.

## 4.4. Conclusions

The results of this study have shown that bare soil by tillage could be a sustainable recommendation for managing the soil, due to the reduced use of agrochemicals and the resulting high yeasts biodiversity. Nevertheless, the presence of herbicides in the vineyard has a minor impact on the diversity of yeast communities associated to grapes. The use of some herbicides (such as glyphosate) could have increased the yeasts populations, including fermentative strains, of which three different genotypes (A, B and C) were found, as a result of its use as a nutrient and as an energetic substrate. Thus, from the fermentative yeasts (like *Saccharomyces*) point of view, in hot and arid environments where the tillage causes degradation on the soils, an alternative recommendation would be the maintenance of bare soil with the use of herbicides. Cover crop in the vineyard increased the albedo, the nutrients and water competition between swards and vine and the fungi population on the floor which may have a competitive effect with grape yeasts populations, reducing their quantity and biodiversity in the vineyard, mainly in fermentative strains.

This is the first step of a study with the aim of evaluate the influence of different floor management strategies in the diversity a distribution of yeast populations associated with the grape berries. Further and deeper studies are necessary in order to reinforce these results.

# Capítulo 5

# EFFECT OF THE ANTIFUNGAL SULFUR AND PENCONAZOLE ON INDIGENOUS YEAST POPULATIONS ASSOCIATED WITH GRAPE BERRIES

*Food Control (Submitted)*

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# Capítulo 5

# 5. Effect of the antifungal sulfur and penconazole on indigenous yeast populations associated with grape berries

***"Esta copa del arca: con azufre la purifica y limpia lo primero, y la lava después con agua pura, él mismo se lavó también las manos y la copa llenó de vino negro"***

- *Extracto del poema 348 de "La Iliada" de Homero.*

Este capítulo afronta la necesidad de conocer cómo influyen dos de los fungicidas más utilizados en viticultura sobre la microbiota de levaduras presentes en las uvas del viñedo. Para ello se llevó a cabo un muestreo durante tres años (2006-2008) con el fin de evaluar la influencia del penconazol, como antifúngico sistémico contra el oídio, y el azufre, como fungicida de amplio espectro. Estos compuestos químicos empleados en agricultura y legales dentro del marco de la normativa vigente de la Unión Europea, se aplicaron en el viñedo en diferentes estadios de desarrollo de la vid, pámpanos de 30-40 cm de diámetro, justo antes de la floración, bayas con tamaño de guisante y bayas con tamaño de garbanzo. Como control se utilizó una parcela del viñedo sin tratar. Al igual que en el *Capítulo 3*, los índices de Ecología clásica fueron una herramienta de utilidad a la hora de tratar e interpretar los datos estadísticamente, utilizándose la riqueza de especies (*S*), el índice de la biodiversidad de Shannon-Wiener (*H'*) y el índice de la dominancia de Simpson (*D*), en lo que a especies identificadas se refiere. Los resultados obtenidos en este estudio confirman que la presencia de fungicidas en el viñedo tiene un efecto negativo sobre las poblaciones de levaduras presentes de forma natural en la uva. El uso de azufre y penconazol en 4 estados fenológicos de la vid afecta significativamente más que cuando estos fungicidas fueron aplicados a dosis más bajas,

solamente en 2 estados de desarrollo. Aún así, el Penconazol mostró un efecto más negativo que el azufre frente a la biodiversidad de levaduras asociadas a la uva. El azufre, además es un antifúngico compatible con las prácticas agrícolas de carácter ecológico.

## Abstract

The aim of this work was to assess the influence of two fungicides at different grapevine growth stages on native yeast populations associated with grape berries. A three year sampling plan was performed to evaluate the effects of penconazole, as systematic antifungal against powdery mildew, and sulfur, as inorganic broad-spectrum pesticide. These agrochemicals were applied in four different vineyard blocks at different grapevine growth stages (bud break, flowering, berries pea-sized and berry touch). An untreated vineyard block was used as control. Classical ecology indexes were calculated to obtain the richness (*S*), the biodiversity (*H'*) and the dominance (*D*) of the species studied. The results of this study showed that the presence of fungicides had negative effects over the abundance and diversity of the species of yeasts identified. Application of sulfur and penconazole at four grapevine growth stages affected significantly yeast communities associated with grape berries. Penconazol had a more negative effect. Sulfur as fungicide management strategy was the best agricultural recommendation at only two grapevine growth stages in order to preserve and enhance yeast biodiversity associated with grape berries. Sulfur applied at flowering and berries pea-sized stages was useful to protect the vineyard against powdery mildew. In addition, this strategy is suitable for both organic and conventional farming systems.

# 5.1. Introduction

The term 'pesticide' covers all agricultural chemicals, such as miticides, insecticides, nematicides, plant growth regulators, herbicides and fungicides that are applied either around or to grape vines. Developments in agricultural technology have continued to keep up with the burgeoning world population, with the proper application of pesticides to control weeds, pests and diseases as important element in this technology (Lee, 1990).

Grapevine powdery mildew (*Uncinula necator*) affects grape berries yield and quality worldwide, and it is recognized as a major disease which results in economic losses in the grape and wine industry (Crisp et al., 2006). In order to control this disease, vine growers of conventional vineyards rely mainly on synthetic fungicides, which have become much more diverse in the last years as demand for new products that specifically target organisms. But these products are generally more expensive than pesticides with broad-spectrum effects, and their cost needs to be justified in terms of positive or null environmental and human health consequences (Thomson & Hoffmann, 2007). Viticulturists involved in organic and biodynamic production do not use synthetic chemicals, the disease is controlled mainly by regular applications of sulfur and copper sulfate, as the sole fungicides allowed in organic production (Comitini & Ciani, 2008).

Most fungicides act directly on essential fungal functions such as cell division, respiration and sterol biosynthesis (Leroux, 2003). Penconazole (1 (4-chlorobenzyl)-1-cyclopentyl-3-phenylurea) is a systematic fungicide against powdery mildew which is considered stable and tends to accumulate in soils and grape berries (Ribeiro et al., 2000; Singh, 2005).

The extensive use of this antifungal in viticulture has resulted into the contamination of surfaces and groundwater (Komárek et al., 2010). This synthetic fungicide acts as an inhibitor of ergosterol biosynthesis present in fungi membranes (Ribeiro et al., 2000), thus it could have negative effects on yeast population. On the other hand, synthetic organic fungicides are banned in European organic viticulture, copper based products, such as $CuCl_2$, Cu $(OH)_2$, $CuSO_4$ and $Cu_2O$, and sulfur-based antifungal are allowed and indispensable for organic vine cultivation at limited doses according to the Commission Directive 2006/60/EC of the European Community. Sulfur is less persistent in the soil environment than penconazole and also it has a lower toxicity to beneficial insects and aquatic species and mammals, including humans (Smith, 2008). It is one of the pesticides permitted as organic pest management strategy.

Numerous studies in the last years have centered on yeast belonging to the genus *Saccharomyces* that are responsible for alcoholic fermentation (Fleet, 2008; Valero et al., 2007). Nonetheless, evidence exists that non-*Saccharomyces* strains may influence the unique enological characteristics of each winegrowing area (Callejón et al., 2010; Francesca et al., 2010) and the presence of fungicides on grapevines can affect yeast metabolic activity (Calhelha et al., 2006; Ganga & Martínez, 2004). A recent study (Cordero-Bueso et al., 2011) showed that the phytosanitary treatments affected grape microbiota negatively, by reducing the number of yeasts isolated and their diversity, principally of fermentative yeast strains. Grape variety and the farming system have a strong influence on the yeast microbiota associated with the vineyard, being the organic vineyard the best option for maintaining a natural reservoir of fermentative yeasts of interest.

The identification of growth stages for the grapevine, as with all crop plants, is essential for decisions on agricultural operations of grapevine growing and diseases control. Thus, in 1995 Coombe proposed a system for identifying grapevine growth stages for all usages, including pesticides use, by comparing the three systems have been described for grapevines: Baggiolini (1952), Eichhorn & Lorenz (1977) and the BBCH system which was adapted by Lorenz et al. in 1994. The latter one has been developed as a model for the European Union. Fungicides usually are applied at different grapevine growth stages such as, bud break, flowering, berries pea-sized and berry touch, these which are more susceptible to *Uncinula necator*.

The aim of this study was to obtain precise information about the influence of different agronomic parameters on the composition and evolution of yeast communities associated with grape berries, by comparing the yeast population density and diversity in grape juice fermentations using grape berries harvested from vineyards with different fungicides management strategies at different grapevine growth stages.

## 5.2. Materials and Methods

### *5.2.1. Sampling plan and fermentation procedure*

This study was performed from 2006 to 2008 in an experimental vineyard with Tempranillo grapevine variety (*Vitis vinifera* L) located in the Madrid winegrowing region, Spain (40° 8' 1.5864" N, -3° 22' 26.9754" W, and 743 m altitude). The climatological data was taken from the vineyard weather station. The mean air temperatures during July, August and September were 23.5 ºC, 23.2 ºC and 23.2 ºC, for 2006, 2007 and 2008 respectively. As regards the mean precipitations for these three

months, the data obtained was 8.3, 6.6, and 27.8 mm. respectively. Grapes were harvested from the vineyard with thirty rows of vines, within which a randomized trial of five block containing three rows with fifteen vines per block on vertical trellises facing the direction of the gradient each one. Between the blocks, the gaps consisted of three untreated rows with fifteen vines each one. The soil management strategy used was bare soil by tillage in all blocks sampled. Irrigation was performed through a drip system, placing a drip every 75 cm and a water flow of 2.2 L/hour, resulting in 150 hours/year. Fungicides against powdery mildew were prepared according to the manufacturer's instructions. These were sprayed, by using a knapsack for each product and taking in account the maximum levels permitted by the European legislation (EU pesticide database, 2010), in four blocks of the vineyard as follows: (SA) micronized sulfur P-300/100 (Cepsul especial 98.5%, Afepasa, DP, 40 kg/Ha) at four grapevine growth stages (bud break (A), flowering (B), berries pea-sized (C) and berry touch (D)), (SB) micronized sulfur P300/100 (Cepsul especial 98.5%, Afepasa, DP, 40 kg/Ha) at two stages (B and C), (PA) Penconazole (Topas 10% p/v, Syngenta AG, Basel, Switzerland; 200 cc/HL) at four stages (A, B, C and D) and (PB) Penconazole (Topas 10% p/v, Syngenta AG, Basel, Switzerland; 200 cc/HL) at two stages (B and C). In a control treatment (CT), vines were not sprayed with fungicides. The safety intervals for the fungicides applied were considered, 25 days for micronized sulfur P300/100 and between 7 and 14 days for Penconazole.

Bunches were always collected from the same plant, facing in the same direction. With the present experimental design, 15 grape samples were collected every year, 3 samples for every applied antifungal treatment. Approximately 2kg of grapes, stems included, were harvested in aseptic conditions from each sampling point and placed directly into

sterile bags, which were transported to the laboratory in portable refrigerators with plastic ice blocks and processed within 2 hours.

At the laboratory, grapes were squeezed by hand in plastic bags, opened in the laminar airflow bench, and 80 mL of juice was poured into 100 mL sterile fermenters. It had two openings, which were blocked with a rubber stopper, the top one with a capillary to allow $CO_2$ to escape. About 50 mL of the must were centrifuged for 5 minutes at 5000 rpm, and the pH of the supernatant was measured with a pH-meter (Crison GLP21, Barcelona, Spain) and Brix degree by refractometry (Atago digital refractometer model CO., LTD. Tokyo, Japan) . The fermenters were carried out at 20 ºC with mechanical agitation (150 rpm). Fermentation progress was monitored daily by loss weight determination.

### *5.2.2. Yeast isolation*

The yeast community present in the fermentation was evaluated when the weight of the must was reduced by 70 g $L^{-1}$, corresponding to the consumption of about two thirds of the sugar content. Ten-fold dilutions of must were spread on YPD plates (yeast extract 1% w/v, meat peptone 1% w/v, glucose 2% w/v and agar 2% w/v) incubated for 24–48 hours and30 colonies were randomly selected.

### *5.2.3. DNA extraction and quantification from isolates*

DNA extraction from yeast isolates was carried out using a commercial kit (ArchivePure DNA Purification System, 5 Prime, Germany), following the instructions provided by the manufacturer, but centrifuging at 14.000 rpm. The DNA was then stored at -20 ºC. An UV-Vis spectrophotometer (Nanodrop 1000, Thermo Fisher Scientific Inc.) was used to calculate the quantity of DNA extracted.

## *5.2.4. Molecular identification of isolates*

- *PCR-RFLP and RAPD-PCR analysis*

The PCR reaction mixes and amplification protocols were the same as those used by Cordero-Bueso et al. (2011). Yeasts were identified to species level by comparing the amplified product and their restriction fragment pattern with those described elsewhere (Esteve-Zarzoso et al., 1999; Fernández-Espinar et al., 2000; Guillamón et al., 1998), and with profiles included in the data base of the Spanish Type Culture Collection (CECT). Also, in each amplification and restriction case, some certified yeast strains (*Torulaspora delbrueckii* CECT1015, *Hanseniaspora guilliermondii* CECT11029, *Metschnikowia pulcherrima* CECT10071, *Pichia toletana* CECT11493, *Pichia anomala* CECT1110, *Saccharomyces cerevisiae* CECT1176, *Kluyveromyces thermotolerans* CECT1962, *Candida sorbosa* CECT11204 and *Candida apicola* CECT11167) obtained from the CECT were used as controls.

- *Microsatellite Multiplex PCR analysis*

The PCR reaction mix and amplification protocols were the same as those used by Vaudano & García-Moruno (2008). Amplified products were separated on an agarose gel (2.5% w/v) with 5 µl $mL^{-1}$ of ethidium bromide (Applichem, USA), in 1X TBE buffer (Sigma-Aldrich, USA) at 100V, for 90 minutes. DNA fragment sizes were determined by comparison with a molecular ladder marker of 100 bp (Promega, Madison, WI, USA).

The banding patterns were processed with cluster analysis software (Bionumerics, Applied Maths, Keistraat, Belgium) using a Dice binary similarity index, and the dendrogram was built with the UPGMA method. Moreover, fragment differentiation and allele size determination was

performed by single capillary automatic electrophoresis in ABI 3130 Genetic Analyzer (Applied Biosystem).

### *5.2.5. Statistical analysis*

Classical Ecology indexes were used to obtain the richness (*S*), the biodiversity (*H'*) and the dominance (*D*) of the species studied following the procedure suggested by Cordero-Bueso et al. (2011). In order to decide whether to accept or reject the null hypothesis, variance analysis (ANOVA) was performed to test the main effects of the factors studied, by means of the SPSS (v.16.0) for windows statistical package.

## 5.3. Results

As a result, Tempranillo grapes were collected from a vineyard, which was treated using four antifungal management strategies described in material and methods.

To get more detailed data, this study was carried out over a period of 3 consecutive years (2006, 2007 and 2008). Every year, 15 grape samples were collected, 3 for each antifungal management strategy studied. The average of pH and the Brix degree of the musts obtained during the 3 years are shown in Table 5.1. Thus, 8, 5, 9, 7 and 7 samples reached spontaneous fermentations in the control (CT), SA, SB, PA and PB respectively. A total of 1080 yeast colonies were isolated when the weight of the must was reduced by 70 g $L^{-1}$, of which 905 yeast strains corresponding to genus of non-*Saccharomyces* and 175 to *Saccharomyces*.

**Table 5.1.** Mean and standard desviation of the ºBrix and pH for the musts obtained from each of the samples collected from the different fungicide management strategies (CT= Control; SA= vineyard block treated with sulfur at grapevine growth stages A, B, C,D; SB= vineyard block treated with sulfur at grapevine growth stages B, C; PA= vineyard block treated with penconazole at grapevine growth stages A, B, C,D; PB= vineyard block treated with penconazole at grapevine growth stages B, C) in 2006, 2007 and 2008 vintages.

| | 2006 | | | | | 2007 | | | | | 2008 | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | CT | SA | SB | PA | PB | CT | SA | SB | PA | PB | CT | SA | SB | PA | PB |
| ºBrix | 24 ± 1 | 25 ± 0 | 25 ± 1 | 24 ± 3 | 23 ± 2 | 28 ± 1 | 28 ± 1 | 25 ± 2 | 25 ± 2 | 26 ± 1 | 26 ± 1 | 26 ± 1 | 26 ± 1 | 25 ± 1 | 26 ± 1 |
| pH | 3.7 ± 0.1 | 3.8 ± 0.0 | 3.8 ± 0.1 | 3.7 ± 0.1 | 3.7 ± 0.1 | 3.5 ± 0.1 | 3.6 ± 0.1 | 3.4 ± 0.1 | 3.4 ± 0.2 | 3.5 ± 0.0 | 3.6 ± 0.1 | 3.4 ± 0.1 | 3.6 ± 0.0 | 3.6 ± 0.1 | 3.6 ± 0.0 |

Over the three years, a large proportion of species were found after fermentation. PCR-RFLP and RAPD-PCR analysis of the isolates obtained from the fermentations showed different profiles for the different species (Table 5.2). The RAPD technique is used to identify yeasts at species level and, occasionally, at strain level. The results obtained permitted the identification of nine different species listed in table 5.2.

Table 5.2. Yeasts species identified in 2006, 2007 and 2008. Size of the PCR products and the restriction fragments of the species obtained with three different endonucleases (*Hae*III*, Cfo*I and *Hinf*I).

| Species | *AP (bp) | Restriction fragments size (bp) | | |
|---|---|---|---|---|
| | | *Hae* III | *Cfo* I | *Hinf* I |
| *Metschnikowia pulcherrima* | 400 | 280 + 95 | 210 + 80 | 190 |
| *Candida apicola* | 490 | 385+88 | 210+184 | 228+133 |
| *Candida sorbosa* | 600 | 600 | 560 | 315 |
| *Pichia anomala* | 630 | 620 | 550 | 310+310 |
| *Hanseniaspora guilliermondii* | 775 | 775 | 340+320+105 | 360+200+160 |
| *Kluyveromyces thermotolerans* | 700 | 300+210+85 | 305+280 | 355 |
| *Torulaspora delbrueckii* | 800 | 750 | 320+210+140+100 | 410+375 |
| *Saccharomyces cerevisiae* | 850 | 325+250+185+150 | 375+325+150 | 375+365+110 |

**AP= 5.8S-ITS amplified product size*

The overall data of the fermentations and diversity of yeasts strains isolated are shown in Table 5.3. *K. thermotolerans*, *M. pulcherrima* and *S. cerevisiae* were the most abundant species in the vineyards with 50.1 %, 16.2 % and 11.4 % respectively. *P. anomala*, *H. guilliermondii* and *C. apicola* were present with 8.1 %, 7.7 % and 5.6 % respectively, and other

species such as *T. delbrueckii* and *C. sorbosa* were found in minor percentages (0.5% and 0.4%).

**Table 5.3.** General data, distribution and diversity measure of the species isolated from spontaneous fermentations of must from grape berries collected from different fungicide management strategies (CT= Control; SA= vineyard block treated with sulfur at grapevine growth stages A, B, C,D; SB= vineyard block treated with sulfur at grapevine growth stages B, C; PA= vineyard block treated with penconazole at grapevine growth stages A, B, C,D; PB= vineyard block treated with penconazole at grapevine growth stages B, C; n.i = no isolates) in 2006, 2007 and 2008 vintages.

| General data and diversity measure | Total three years | | | | | Vintage 2006 | | | | | Vintage 2007 | | | | | Vintage 2008 | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | CT | SA | SB | PA | PB | CT | SA | SB | PA | PB | CT | SA | SB | PA | PB | CT | SA | SB | PA | PB |
| Grape berries samples | 9 | 9 | 9 | 9 | 9 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 |
| Spontaneous fermentations | 8 | 5 | 9 | 7 | 7 | 3 | 3 | 3 | 3 | 3 | 3 | 2 | 3 | 3 | 3 | 2 | 0 | 3 | 1 | 1 |
| Total number of species (S) | 6 | 5 | 5 | 3 | 5 | 4 | 2 | 3 | 3 | 3 | 5 | 4 | 5 | 2 | 3 | 2 | 0 | 2 | 1 | 1 |
| *Metschnikowia pulcherrima** | 30 | 31 | 7 | 50 | 5 | 0 | 0 | 0 | 30 | 0 | 30 | 31 | 7 | 20 | 5 | 0 | 0 | 0 | 0 | 0 |
| *Candida apicola** | 32 | 0 | 0 | 0 | 29 | 30 | 0 | 0 | 0 | 29 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Candida sorbosa** | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Pichia anomala** | 42 | 0 | 46 | 0 | 0 | 12 | 0 | 30 | 0 | 0 | 30 | 0 | 16 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Hanseniaspora guilliermondii** | 0 | 60 | 23 | 0 | 0 | 0 | 60 | 0 | 0 | 0 | 0 | 0 | 23 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Kluyveromyces thermotolerans** | 84 | 51 | 104 | 130 | 172 | 30 | 30 | 30 | 30 | 60 | 24 | 21 | 14 | 70 | 82 | 30 | 0 | 60 | 30 | 30 |
| *Torulaspora delbrueckii** | 0 | 2 | 0 | 0 | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 3 | 0 | 0 | 0 | 0 | 0 |
| *Saccharomyces cerevisiae* | 48 | 6 | 90 | 30 | 1 | 18 | 0 | 30 | 30 | 1 | 0 | 6 | 30 | 0 | 0 | 30 | 0 | 30 | 0 | 0 |
| Total number of individuals (N) | 240 | 150 | 270 | 210 | 210 | 90 | 90 | 90 | 90 | 90 | 90 | 60 | 90 | 90 | 90 | 60 | 0 | 90 | 30 | 30 |
| Shannon-Wiener index (*H'*) | 1.60 | 1.25 | 1.34 | 0.92 | 0.61 | 1.32 | 0.64 | 1.1 | 1.1 | 0.68 | 1.31 | 1.06 | 1.51 | 0.53 | 0.36 | 0.69 | n.i | 0.63 | 0 | 0 |
| Simpson's index (*D* ) | 0.23 | 0.32 | 0.30 | 0.47 | 0.69 | 0.28 | 0.55 | 0.33 | 0.33 | 0.54 | 0.30 | 0.40 | 0.24 | 0.65 | 0.83 | 0.50 | n.i | 0.55 | 1 | 1 |

*total number of isolates of each species

All strains identified as *S. cerevisiae* were genotyped by microsatellite multiplex PCR analysis using SC8132X, YOR267C and SCPTSY7 primers. Four different electrophoretic patterns (A, B, C and D) were obtained (Table 5.4).

**Table 5.4.** Allele size obtained by single capillary automatic electrophoresis of the 175 *Saccharomyces cerevisiae* strains identified.

| Genotype | Number of *S. cerevisiae* | Allele size (bp) | | | | | |
|---|---|---|---|---|---|---|---|
| | | SCPTSY7-1 | SCPTSY7-2 | SC8132X-1 | SC8132X-2 | YOR267C-1 | YOR267C-2 |
| A | 30 | 292 | 292 | 212 | 310 | 308 | 389 |
| B | 114 | 261 | 312 | 155 | 212 | 389 | 389 |
| C | 30 | 271 | 271 | 206 | 206 | 389 | 389 |
| D | 1 | 256 | 271 | 206 | 231 | 389 | 413 |

Distribution of the different genotypes in the vineyard block during the three years studied are shown in Table 5.5.

**Table 5.5.** Distribution of different genotypes (A, B, C, D) of *S. cerevisiae* in the different antifungal management strategies strategies (CT= Control; SA= vineyard block treated with sulfur at grapevine growth stages A, B, C,D; SB= vineyard block treated with sulfur at grapevine growth stages B, C; PA= vineyard block treated with penconazole at grapevine growth stages A, B, C,D; PB= vineyard block treated with penconazole at grapevine growth stages B, C) in 2006, 2007 and 2008.

| Genotype | CT | SA | SB | PA | PB |
|---|---|---|---|---|---|
| Total three years | | | | | |
| A | 30 | 0 | 0 | 0 | 0 |
| B | 18 | 6 | 30 | 60 | 0 |
| C | 0 | 0 | 30 | 0 | 0 |
| D | 0 | 0 | 0 | 0 | 1 |
| Vintage 2006 | | | | | |
| B | 18 | 0 | 30 | 30 | 0 |
| D | 0 | 0 | 0 | 0 | 1 |
| Vintage 2007 | | | | | |
| B | 0 | 6 | 0 | 30 | 0 |
| Vintage 2008 | | | | | |
| A | 30 | 0 | 0 | 0 | 0 |
| C | 0 | 0 | 30 | 0 | 0 |

Taking into account the results obtained in this study and regarding the yeasts' richness (*S*), six different species were isolated from the

untreated control samples, while five species were found in SA, SB and PB. Only four species were represented in PA (Table 5.3). The general index of biodiversity Shannon-Wiener (*H'*) and the concentration of dominance of Simpson (*D*) were calculated on the basis of the number of identified species (Table 5.3). The musts obtained from the untreated vineyard block seemed to have the highest biodiversity of yeasts (*H'*= 1.60), and the lowest concentration of dominance (*D*= 0.23). Shannon's index obtained for musts from grape berries collected at SA and SB were *H'*= 1.25 and *H'*= 1.34 respectively, and Simpson's index were *D*= 0.32 and *D*= 0.30.The *t-Student* applied to the Shannon-Wiener indexes showed that the differences of the means were not significant with respect to the yeast communities in the musts obtained from the untreated samples (CT). Conversely, the yeast strains identified from musts obtained from PA and PB exhibited the lowest Shannon's index *H*'= 0.92 and *H*'= 0.61 and the highest dominance index *D*= 0.47 and *D*= 0.69, respectively (Table 5.3). Shannon-Wiener indexes for SA and SB were significant different ($p<0.05$).

Furthermore, the Shannon's index and the concentration of dominance (*D*) were calculated for *S. cerevisiae* on the basis of the number of different genotypes found. The highest biodiversity of strains (*H'*= 0.66), and the lowest concentration of dominance (*D*= 0.53) was found on the untreated vineyard block (CT); similar results were found in the SB (*H*'= 0.64) and (*D*= 0.55). On the contrary, the rest of the vineyard blocks (SA, PA and PB) exhibited the lowest Shannon's index (*H*'= 0) and the highest dominance index (*D*= 1). The *t-Student* test applied to the Shannon-Wiener indexes showed that the differences were significant with $p< 0.05$ for the SA, PA and PB with respect to the CT. For a better understanding of the results, these have been divided according to the three vintages sampled during 2006, 2007 and 2008.

### *5.3.1. Vintage 2006*

All 15 samples collected from 5 different stages in 2006 completed spontaneous fermentations (Table 5.3). A total of 450 colonies were isolated after 2/3 of the fermentable sugars have been consumed (371 non-*Saccharomyces* strains and 79 *Saccharomyces*).

According to the quantitative analyses, six species were isolated in this vintage and which showed differences throughout the antifungal treatments. The higher richness (S) was observed in the untreated vineyard block (CT) (Table 3). *K. thermotolerans* was the predominant non-*Saccharomyces* species in grape must obtained from all vineyard blocks. The more relevant results were the absence of *M. pulcherrima* and *H. guilliermondii* in the control (CT). The highest biodiversity (*H'*= 1.32), and the lowest concentration of dominance (*D*= 0.28) was found on the untreated vineyard block (CT). The SB and PA treatments presented the same results (*H*'**=** 1.1) and (*D*= 0.33). The vineyard blocks (SA and PB) showed the lowest Shannon's index and the highest dominance index (Table 5.3), which were significantly different with $p< 0.05$ with respect the control.

Regarding the source of fermentative yeast species, the results obtained in this study show that only in the SA no *S. cerevisiae* strains were found. However, a total of 18 fermentative yeast strains were isolated in musts from the CT, and another 30 colonies of *Saccharomyces* in both SB and PA. After the microsatellite multiplex PCR analysis all isolates were found to belong to the genotype B. One unique yeast strain of the genus *Saccharomyces* was found in musts from grapes berries collected in the PB.

### *5.3.2. Vintage 2007*

Fifteen samples harvested reached spontaneous fermentations except for one of the samples from the SA. 420 yeast colonies were isolated of which 9% belonged to *Saccharomyces* species and 91% to strains of the genus of non-*Saccharomyces* (Table 5.3).

*C. apicola* and *C. sorbosa* were only found in the CT. *K. thermotolerans* and *M. pulcherrima* were the most abundant species during 2007, while *H. guilliermondii* and *T. delbrueckii* were only isolated in treated vineyard blocks (Table 5.3). *S. cerevisiae* represented 9 % of the total of isolates from the spontaneous fermentations of the musts obtained from the vineyard blocks treated with sulfur at the different stages in 2007. Contrary to what was expected, *S. cerevisiae* strains were not isolated in the vineyard control (CT). Only one genotype (B) was described within the *Saccharomyces* analyzed in 2007 (Table 5). Regarding the ecological indexes, the CT and the SB showed the highest biodiversity and the lowest concentration of dominance (Table 5.3).

### *5.3.3. Vintage 2008*

A total of 15 grape samples collected from the different vineyard blocks treated with different antifungal strategies, of which 2, 3, 1 and 1 samples reached spontaneous fermentations in the CT, the SB, the PA and the PB respectively. No spontaneous fermentations occurred in the SA. A total of 210 colonies were isolated from the fermentations, 150 corresponding to *K. thermotolerans* and 60 isolates were *S. cerevisiae* (Table 5.3). In 2008, species richness (S) was very low with respect the other two previous years, only two species identified from all fermentations of the musts from each vineyard block (Table 5.3).

*Saccharomyces* yeasts were found in must from the vineyard block managed with sulfur at the grapevine growth stages (B, C), and in spontaneous fermentations from the untreated block. Confirming the preliminary findings, the most evident results are the values of the Shannon-Wiener and Simpson's indexes showing once again the highest biodiversity and the lowest concentration of dominance in the CT and the SB (Table 3). By the way, two different genotypes (A, C) were identified by microsatellite multiplex PCR (Table 5.5).

## 5.4. Discussion

The results obtained in this study show the occurrence of different species of *Saccharomyces* and non-*Saccharomyces* in function of the treatment with the fungicides penconazole and sulfur in specifics stages of the vegetative development of grape berries. The presence of the identified species in this work has also been described in other surveys of yeast communities in wine (Callejón et al., 2010; Cordero-Bueso et al., 2010; Esteve-Zarzoso et al., 1998; Pretorius, 2000; Ribéreau-Gayon et al., 2006; Romancino et al., 2008). In all vintages (2006, 2007 and 2008), a large proportion of non-*Saccharomyces* were found. In our study these strains represented 84% of the total yeasts isolated over the three years. These data confirm previous reports indicating that *S. cerevisiae* is not present in a large numbers on vineyards (Cordero-Bueso et al., 2011; Mortimer and Polsinelli, 1999; Pretorius 2000). In this work, a total of 175 native *S. cerevisiae* strains were isolated. These results clearly indicate that indigenous fermentative yeasts occur in a very low percentage (16%) in vineyard ecosystems of the Madrid winegrowing region.

Reports of harmful influence of fungicides to yeasts associated with the grape berries are numerous in laboratory studies (Čadež et al., 2010; Calhelha et al., 2006; Conner, 1983; Ribeiro et al., 2000). In the other hand, disturbances also may determine the species diversity in field studies (White & Pickett, 1985). High exposures to ultraviolet radiation (Smart, 2002), high temperatures and rainfall (such as rainstorms) and the airflow speeds (Maltam, 2008) are examples of disturbance. Thus, the effects of the weather conditions and nutritional factors in open field experiments cannot be avoided.

In our study, the mean air temperatures 23.5 ºC, 23.2 ºC and 23.2 ºC for 2006, 2007 and 2008 respectively were normal during the three months of vintage, but the mean precipitation during July, August and September of 2008 was slightly higher, as well as stormy, compared with the same period in 2006 and 2007. Accordingly, the yeast density, species richness and biodiversity differed significantly between 2008 and the other two seasons (2006 and 2007). In the 2008 vintage, the number of spontaneous fermentations reached was lower than in 2006 and 2007, and only two species (*K. thermotolerans* and *S. cerevisiae*) were isolated. Excess of water causes exosmosis and weakness in the skin and some juice might escape. The presence of a rich sugary medium on the grape surface could increase the proportion of some yeast species with the capacity to compete for sugars such as *S. cerevisiae* (Čadež et al., 2010; Combina et al., 2005; Gildemacher et al., 2006; Longo et al., 1991).

Furthermore, the fungicide mode of action may be non-specific and might act on organisms other than the target hanger-on (Calhelha et al., 2006). The doses applied at four grapevine stages development affected notably the abundance and diversity of yeast populations obtained from spontaneous fermentations of the grapes collected. The fungicide

treatments with penconazole exerted significant effects on the yeast communities with respect to the control over the 3 years. This is in agreement with the described activity of penconazole as an inhibitor of ergosterol biosynthesis in fungi (Ribeiro et al., 2000). Interestingly, we observed an absence of *M. pulcherrima* and *H. guilliermondii* in the untreated vineyard block in 2006. However, these species appeared in the PA and in the SA treatments, respectively. Several authors (Čadež et al., 2010; Comitini & Ciani, 2008) observed that *H. uvarum*, *H. guilliermondii* and *M. pulcherrima* were resistant species for some fungicides such as pyrimethanil, fludioxonil and cyprodinil. These results are in accordance with previous reports on the influence of fungicides on population abundance, richness and diversity (Calhelha et al., 2006; Comitini and Ciani, 2008; Gildemacher et al., 2004). The vineyard block treated with sulfur at the B and C grapevine growth stages during all vintages was statistically not significant with respect to the untreated block. Therefore, these results indicate that SB might be an agricultural recommendation in order to preserve and enhance the yeast biodiversity associated with grape berries.

Regarding yeast source of *S. cerevisiae*, in their natural environment, these yeasts have to cope also with changing temperature, humidity and fungicides. To test whether the prevailing *Saccharomyces* yeast communities were present in the different antifungal management strategies, spontaneous fermentations were undertaken in order to obtain a significant population of these yeast, because of its lower abundance on grapes. These samples yielded 175 colonies identified as indigenous *S. cerevisiae* among which we found four different genotypes (A, B, C and D). The genotype B was the most abundant through the untreated vineyard block, and the different treatments in 2006 and 2007 (being mostly abundant in the SB) except in the PB, which showed an exclusive

genotype (D) in 2006. Genotypes A and C were only found in spontaneous fermentations of musts from grapes collected in 2008 in the vineyard block treated with sulfur at two grapevine development stages and in the untreated one. This last observation may be confirming a succession on yeast communities associated with grape berries and the disturbances hypothesis stated before. Contrary to the results obtained by Comitini & Ciani (2008) that suggested fungicides cause the elimination of the fermenting ascomycetous yeast on grapes berries, we believe that our results provide strong evidence for a discrete population of fermentative yeasts residing in the sampled vineyard blocks.

## 5.5. Conclusions

On the basis of the above findings, it is concluded that the presence of fungicides exert negative effects over the abundance and diversity of the majority of the species. Penconazole was the most negative antifungal. Furthermore the application of both fungicides (sulfur and penconazole) at four grapevine growth stages affect significantly yeast communities associated with grape berries. Thus, using sulfur at only two grapevine growth stages as fungicide management strategy is best agricultural recommendation in order to preserve and enhance the yeast biodiversity associated with grape berries besides protecting the vineyard against powdery mildew. In addition, this strategy is suitable for both organic and conventional farming systems.

Our results provide strong evidence for a discrete population of fermentative yeasts residing in the sampled vineyard blocks. In addition, the vineyard treated with sulfur at flowering and berries pea-sized stages showed the higher abundance and diversity of *S. cerevisiae* genotypes and this was also the case for the untreated vineyard.

# Capítulo 6

# REMANENCE AND SURVIVAL OF COMMERCIAL YEAST IN DIFFERENT ECOLOGICAL NICHES OF THE VINEYARD

*FEMS Microbiology Ecology (2011). doi: 10.1111/j.1574-6941.2011.01124.x.*

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# Capítulo 6

# 6. Remanence and survival of commercial yeast in different ecological niches of the vineyard

***"La vida es un instinto de desarrollo, de supervivencia, de acumulación de fuerzas, de poder"***

- *Cita del filósofo alemán Friedrich Wilhelm Nietzsche.*

Las bodegas son sistemas abiertos y una vez finalizada la vinificación, las levaduras son eliminadas en gran cantidad junto con las aguas residuales y orujos procedentes de la vinificación, dispersándose éstas en el medio ambiente. El comportamiento ecológico de estas levaduras en el viñedo continúa hoy siendo escaso, así, es necesaria la aportación de nuevos datos que nos ayuden a evaluar los riesgos ambientales asociados a la utilización de estas levaduras. En este capítulo se ha estudiado la evolución y seguimiento de una levadura comercial (K1M, Lalvin) diseminada de forma voluntaria en el viñedo, con el objetivo de evaluar su capacidad de supervivencia y permanencia en el mismo. Se llevó a cabo un extenso muestreo durante 36 meses en un viñedo de la Comunidad de Madrid, se tomaron muestras de uva, suelo, hoja y tronco en la zona del viñedo diseminada y a diferentes distancias del punto de diseminación. La levadura diseminada estuvo bien representada en el viñedo durante los primeros 8 meses. Después de 24 meses, no se obtuvo ninguna evidencia de que dicha levadura permaneciera en el viñedo integrándose como parte de la microbiota de éste. No obstante, una pequeña población de la levadura comercial se encontró a 50 metros del área diseminada. A los 36 meses, no se encontró ninguna levadura comercial en las muestras analizadas en todo el viñedo. La uva y el suelo fueron los principales reservorios de la levadura comercial durante el periodo de envero y maduración del fruto,

sin embargo durante la fase de reposo vegetativo la mayor presencia de levaduras se encontró en el tronco. Los resultados obtenidos tras el análisis de la variación de las poblaciones de levaduras año tras año indican que no se produjo la colonización de la levadura diseminada en el viñedo y por tanto, su presencia estuvo limitada en el tiempo y en el espacio.

## Abstract

The use of commercial wine yeast strains as starters has been extensively generalised over the past three decades. Wine yeasts are annually released in winery environments; however, little is known about the fate of these strains in the vineyard. To evaluate the industrial starter yeasts' ability to survive in nature and become part of the natural microbiota of musts, commercial yeast was disseminated voluntarily in an experimental vineyard in the Madrid region (Spain). A large sampling plan was devised over three years, including samples of grapes, leaves, bark and soil. The disseminated yeast was well represented in the vineyard during the first 8 months. After two years the commercial yeast strain had not survived in the sprayed plants, but a residual population was found in plants situated 50 metres east of the sprayed area. After three years, commercial yeast disseminated was not found in the sampled vineyard. Grapes and soil showed the highest number of yeasts isolated in the vegetative period, the bark being the main natural reservoir during the resting stages. The result of analysis of population variations from year to year indicated that permanent implantation of commercial strain (K1M) in the vineyard did not occur and its presence was limited in time.

# 6.1. Introduction

The use of commercial wine yeasts strains as starters has grown extensively over the past three decades. Today, the majority of wine production is based on the use of active dried yeast, which ensures rapid and reliable fermentations, and reduces the risk of sluggish or stuck fermentations and of microbial contaminations. Most commercial wine yeast has been selected in the vineyard for oenological traits such as fermentation performance, ethanol tolerance, absence of off-flavors and production of desirable metabolites. These and other technological developments have contributed to improve wine quality, and have enhanced the ability of winemakers to control the fermentation process and achieve specific outcomes.

On the other hand, and as a result of modern winemaking practices and diversification of wine products, there is an increasing quest for specialised wine yeast strains. Recombinant-DNA technologies have been successfully applied to wine yeast, generating specialized wine yeast strains which have been engineered for specific traits, such as improved fermentation performance and process efficiency, wine sensory quality and health benefits for consumers. Recent advances in the yeast selection tending towards genetic engineering have provoked much discussion. Consumers' and governments' concern about public health and the environmental safety of microbial strains engineered by recombinant DNA technologies remains a hurdle to the commercial use of these yeasts (Schuller and Casal, 2005; Verstrepen et al. 2006; Fleet, 2008). Wine yeast strain development is an important source of new genetic diversity to increase the options available to winemakers. Consumer demands for newer styles of wines and increasing concerns about the environmental consequences of wine production are providing

new challenges for innovation in wine fermentation technology (Pretorius and Hoj, 2005; Fleet, 2008). A list of commercially available strains of wine yeasts has been compiled by Henschke (2007). Nevertheless, an important question is if the use of these microorganisms could have a real environmental impact.

Wineries are open systems and commercial yeasts are used without any special control and could therefore be dispersed into the environment in large quantities. The behaviour of these yeasts in the grape berries was studied by several authors (Comitini and Ciani, 2006; Schuller et al. 2005; Valero et al. 2005, 2007; Goddard et al. 2010). In these studies commercial yeasts were disseminated into the environment year by year and it was not possible to determinate their real permanence in the vineyard. This study aims to complete this data by determining the infiltration and permanence in time of the disseminated yeast population. Similarly, as the studies mentioned analyse the populations present in grapes, we do not know if the soil or any other part of the plant could act as a natural reservoir for commercial yeasts. Therefore the permeating of commercial yeast in other vineyard niches, such as bark, leaf and soil is totally unknown, as well as their potential impact on the autochthonous microbiota. In particular, it is not known if commercial dry yeasts are able to survive in non-grape berry niches, nor whether they will become members of the must microbiota the following years. On the other hand, the long-term survival on a spatial scale of a community of commercial strains can be influenced by numerous factors: self fertilisation (Cubillos et al. 2009), nutrient quantities in the different parts of a vineyard (Palková and Váchová, 2006), dissemination vectors such as insects, small mammal and human activities (Goddard et al. 2010), soil type and water run-off (Valero et al., 2005; Marques et al., 2009), the age of the vineyard (Pretorius, 2000) environmental adaptation to a new geographic

area (Salinas et al. 2010) and the continuous changing of climatic conditions such as rainfall, wind direction, temperature, etc. (Pretorius et al. 2000; Schuller et al., 2005; Valero et al. 2005, 2007; Francesca et al. 2010).

This study aims to evaluate the behaviour of commercial yeast in the environment via the voluntary dissemination of *S. cerevisiae* K1M (Lallemand, France) into different ecological niches of the vineyard in order to answer questions such as, can commercial yeasts remain in the vineyard permanently and form part of the autochthonous microbiota ? or, is there an ecological niche that acts as the natural reservoir of these yeasts? amongst others. This information will be highly useful for the continuity of the oenological practice of the use of selected yeasts and even for the possible use of yeasts obtained via new technologies.

## 6.2. Materials and Methods

### *6.2.1. Sampling plan and fermentation procedure*

This study was carried out over three years (2006-2009), in an experimental vineyard with Mazuelo, Cabernet Sauvignon, and Prieto Picudo, (*Vitis vinifera* L) grapevine varieties located in Alcalá de Henares in the Madrid winegrowing region, Spain (40° 31' N, 3° 17' W, 610 m altitude). The climatological data was obtained from the vineyard weather station. For these four years the data was fairly homogeneous, and read as follows: for 2006, 2007, 2008 and 2009 the annual mean air temperature was 15.4ºC, 13.4ºC, 14.1ºC and 15.2ºC respectively. Regarding the annual mean for precipitation, the data obtained was 414.01 mm, 429.71 mm, 484.90 mm and 468.11 mm. Under high pressure, the dominant wind direction in the sampling area is from north-

east (NE) to south-west (SW). The grapes were harvested in an experimental vineyard with vertical trellises facing in the direction of the gradient with Guyot pruning and bare soil by tillage. Irrigation was performed through a drip system, placing a drip every 75cm, with a water flow of 2.2 L/hour, resulting in 150 hours/year. The soil is a typical Henares river terrace. It is characterised by non-saturated water at any time of year and by the presence of large cracks in dry seasons. According to Soil Survey Staff (1999), the soil was classified as follows; Calcic Haploxeralf Alfisol (FAO (2007) equivalence; Calcic Luvisol) with a variable depth of 0, 14, 32 and 60 centimetres. The soil composition was 44% sand, 35% silt, 20% clay and 1% organic matter. The pH obtained was 8.0.

In order to evaluate the evolution of commercial yeast in the environment, a cell suspension of the dry active commercial yeast K1M (Lallemand, France) was prepared to obtain a concentration of $1 \times 10^6$ cells $ml^{-1}$ approximately and each stump or plant and the soil around it were sprayed with a litre of this cell suspension, at the beginning of harvest in the region. The study was carried out over a period of 36 consecutive months starting with the 2006 harvest until the 2009 campaign. Three grapevine plants were sampled within the sprayed area (25 meter radius). The subsequent samples were always collected from the same vine. With the present experimental design, samples of grape, leaf, soil and bark were collected at the following times: 0 (on the same day of the dissemination) and 7 days, and 1, 3, 6, 8, 12, 18, 24 and 36 months after dissemination. A control sampling was performed before the dissemination. In order to find out if sprayed yeasts were disseminated to other points in the vineyard, additional samples were collected over 25 metres around the dissemination point, for the control sample, and from 0 to 18 months. After 24 and 36 months, additional samples were collected

at specific distances of 25, 50 and 100 metres to the North, West, East and South of the point of dissemination. This sampling plan is shown in Figure 6.1. Approximately 2 kg of grapes, stems included, and 5 g of leaf, soil and bark were collected in aseptic conditions from each sampling point and placed directly into sterile bags, which were transported to the laboratory under refrigeration conditions and processed within the following 2 hours.

At the laboratory, grapes were squeezed by hand in the plastic bags. These bags were opened in the laminar airflow bench, and 80 mL of juice was poured into 100 mL sterile fermenters. Another 50 mL of the must were centrifuged for 5 minutes at 17734 x g, the supernatant was taken to measure its pH with a pH meter (Crison GLP21, Barcelona, Spain) and Brix degree by refractometry (Atago digital refractometer model CO., LTD. Tokyo, Japan) .

In order to dislodge the yeasts from the leaf, soil and bark samples, 1 g of each were transferred into an assay tube (30 x 115 mm) containing 25 ml of sterile 0.9% sodium chloride solution. The tubes were shaken on a Mixer Vortex vigorously at high speed for 60 seconds to mix the sodium chloride solution with the yeasts. The mix samples were poured into 100 mL sterile fermenters containing 55 mL of synthetic must, MS medium, which mimics a standard grape must (Bely et al, 1990), the pH of MS medium is adjusted to 3.3 by NaOH.

The fermenters with 80 mL of must were placed in a controlled temperature chamber at 20ºC with mechanical agitation. Fermentation progress was monitored daily by loss weight determination.

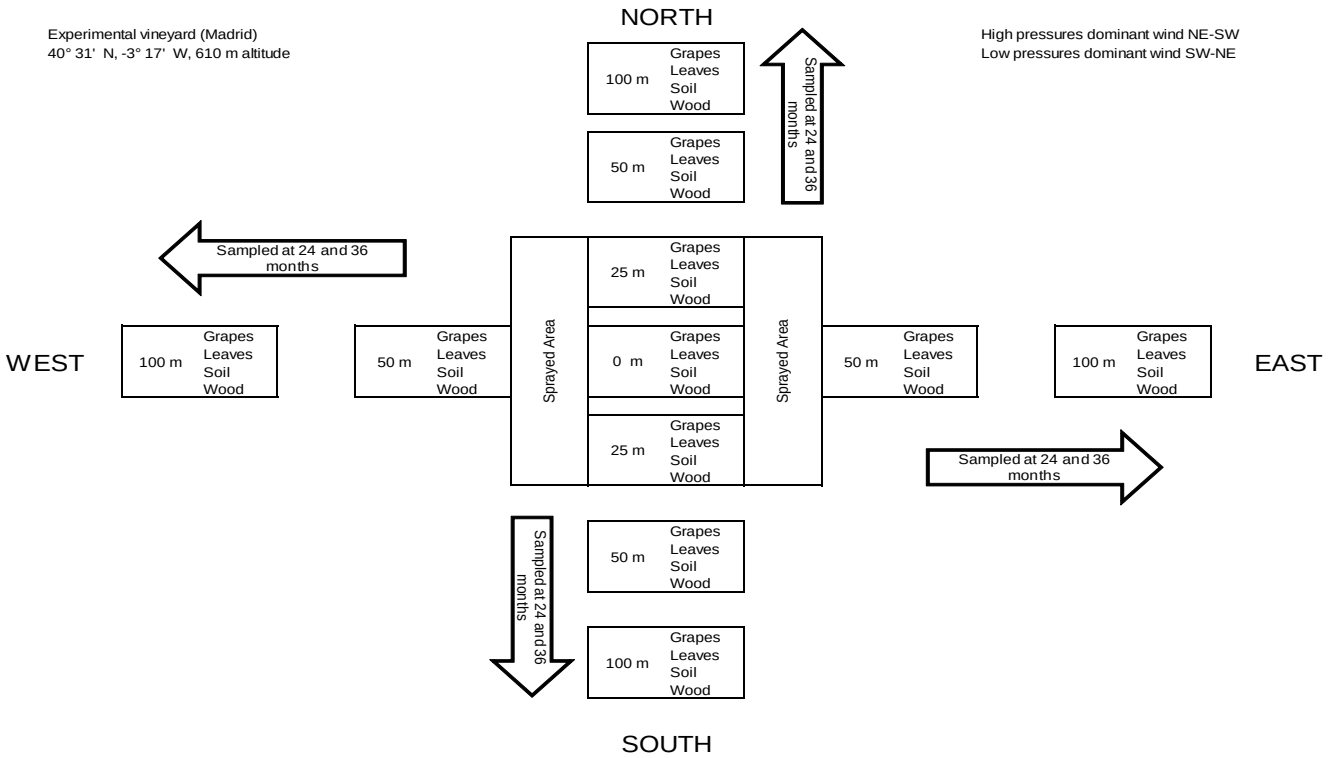



**Figure 6.1. Map of the experimental vineyard located in Alcalá de Henares, in the Madrid winegrowing region**

### 6.2.2. Yeast isolation

The yeast community present in the fermentation was evaluated when the weight of the must was reduced by 70 g $L^{-1}$, corresponding to the consumption of about two thirds of the sugar content. Ten-fold dilutions of 100µl of must were spread on plates with YPD medium (yeast extract 1% w/v, meat peptone 1% w/v, glucose 2% w/v and agar 2% w/v). The plates were incubated at 28ºC for 24 – 48 hours to allow their growth. After that, colonies were counted, and 30 colonies were randomly selected from each fermentation sample.

L-lysine agar (Barnett et al., 2000), which is unable to support the growth of *S. cerevisiae*, was used to assess the non-*Saccharomyces* yeasts. All isolates that were not able to grow on the YNB medium with L-lysine as the sole nitrogen source, but grew on the control medium YNB with ammonium sulfate were considered as *S. cerevisiae* and selected for further steps.

### 6.2.3. Saccharomyces cerevisiae (K1M) detection

The commercial yeast strain of *S. cerevisiae* K1M (Lallemand, France) was used to monitor the persistence and the evolution of commercial yeast in the vineyard. This yeast has two resistance markers, to the herbicide Diuron and to the antibiotic Erythromycin. Although, it was isolated in the south east of France, it is not autochthonous of the area under study and it was not used either in the area or within a radius of 60-80 km, as there are not wineries in the vicinity. Furthermore, to the best of our knowledge, this yeast has never been used in wineries in the Madrid region.

In order to select K1M strains, the isolates were grown in a selective medium N+E+D. N medium contains yeast extract 1% w/v, meat peptone 1% w/v, glycerol 2% w/v and agar 2% w/v diluted in 1 L of Sörensen buffer 0.05 M, pH 6.25, plus Diuron, minimum 98% (Sigma-Aldrich Chemie GmbH, Germany) 0.08% w/v diluted in 2.5 ml of acetone (Merck KGaA, Germany) and Erythromycin (Sigma-Aldrich Chemie GmbH, Germany) 0.1% w/v diluted in 4 ml of absolute ethanol (Merck KGaA, Germany). A medium without Diuron and Erythromycin was used as negative control.

### *6.2.4. Molecular identification*

A current molecular biology technique, microsatellite multiplex PCR (Vaudano and García-Moruno, 2008), was used in order to confirm the results obtained with the traditional plate-count technique by using the selective medium. Colonies isolated from N+E+D medium were assayed to verify whether they had the same microsatellite pattern as *S. cerevisiae* strain K1M (Lallemand).

DNA extraction from yeast isolates was carried out using a commercial kit (ArchivePure DNA Purification System, 5 Prime, Germany), following the instructions provided by the manufacturer, but centrifuging at 17734 x g. The DNA was then stored at -20ºC. An UV-Vis spectrophotometer (Nanodrop 1000, Thermo Fisher Scientific Inc.) was used to calculate the quantity of DNA extracted, covering a spectral range from 220 to 750nm.

Strains were genotyped with three microsatellite loci, SC8132X, YOR267C and SCPTSY7. They were used because of their high degree of polymorphism (Field and Wills, 1998; González-Techera et al. 2001; Vaudano and García-Moruno, 2008). The PCR reaction mix and the

amplification protocols followed were the same as those used by Vaudano & García-Moruno (2008). Amplified products were separated on an agarose gel (2.5% w/v) with 5 µl/mL of ethidium bromide (Applichem, USA), in 1X TBE buffer (Sigma-Aldrich, USA) at 100V for 120 minutes. DNA fragment sizes were determined by comparison with a molecular marker (100pb ladder, Promega, Madison, WI, USA). Moreover, fragment differentiation and allele size determination were performed by single capillary automatic electrophoresis in ABI 3130 Genetic Analyzer (Applied Biosystem). In order to determinate whether the patterns obtained from isolated yeast were identical to sprayed yeast, they were compared with the pattern of the commercial yeast strains K1M (Lallemand).

## 6.3. Results

The aim of this work is to determine the presence and permanence in the vineyard of a population of commercial wine yeast disseminated in the vineyard to know their impact on the vineyard microbiota. The climatological conditions and other agronomic parameters, such as pruning, irrigation, soil type, etc. are indicated in Materials and Methods. In order to obtain more detailed information about the persistence and implantation of commercial yeast in the environment, K1M strains (Lallemand) were sprayed on an experimental vineyard in the Madrid winegrowing region (Spain). This commercial yeast was isolated in the Languedoc region of France and was never found as autochthonous yeast in Spain. The vineyard sprayed was sampled over the next three years, in the area of dissemination and at different distances from this area.

A total of 172 samples were collected over 36 months; 37 of which were taken from grapes, 37 from leaves, 49 from bark and 49 from soil. A

sample in triplicate of grapes, leaves, bark and soil respectively was taken before commercial yeast dissemination and was used as control. The first year of the study, 12 samples, 3 for each ecological niche (grape, leaf, bark and soil), were collected after 0, 7 days, 1 month and 12 months. Also during the period when there are no grapes or leaves on the vine, 3, 6, 8 months and 18 months, a total of 6 samples (3 from the bark and 3 from the soil) were collected. Lastly to evaluate the distribution and evolution of K1M strains and because several chemical, physical and biotic factors could influence the dissemination; at 24 and 36 months, the sampling plan was also extended to 25, 50 and 100 metres from the initial area of dissemination (Figure 1). Hence, at both times a total of 11 samples were collected from each part of the vine (grape, leaf, soil and bark).

The musts obtained from samples of grape berries were in optimal conditions to carry out the spontaneous fermentations in the presence of fermentative microorganisms. The pH and the Brix degree of the musts obtained are shown in Table 6.1. Fermentation of the collected samples from the soil and other parts of the vine were carried out in synthetic must, mimicking a standard grape must, as indicated in Material and Methods. Fermentation processes were used as an enrichment method or selection of fermentative strains such as K1M. This method does not reflect the exact initial population in the vineyard, which will always be less in the vineyard than after fermentation. This method was used in order to ensure the detection of fermentative yeast present in very low numbers in the vineyard (Mortimer and Polsinelli, 1999; Pretorius 2000; Martini, 2003).

**Table 6.1.** Brix degree and pH for 23 musts obtained from the experimental vineyard during 36 months of the study (Mean ± S.D.).

| | Control | Time 0 | 1 Week | 1 Month | 3 Months | 6 Months | 8 Months | 12 Months | 18 Months | 24 Months | 36 Months |
|---|---|---|---|---|---|---|---|---|---|---|---|
| º Brix | 24.6 ± 0.4 | 24.9 ± 0.6 | 24.9 ± 0.6 | 25.2 ± 1.3 | - | - | - | 26.2 ± 0.1 | - | 22.0 ± 2.8 | 26.9 ± 2.1 |
| pH | 3.4 ± 0.0 | 3.8 ± 0.0 | 3.7 ± 0.1 | 3.5 ± 0.0 | - | - | - | 3.6 ± 0.0 | - | 3.5 ± 0.1 | 3.7 ± 0.2 |

From the total of 172 samples collected, 72 completed spontaneous fermentations under laboratory conditions, of which 23 were from grapes, 11 from leaves, 18 from the bark and 20 from soil. Thirty colonies were randomly selected from each of the 72 samples which reached spontaneous fermentations. Thus, a total of 2160 yeast colonies were isolated. Based on the L-lysine utilisation method (Barnett el al. 2000), 1336 were non *Saccharomyces* yeasts (435, 188, 360 and 353 from grapes, leaves, bark and soil respectively) and 824 *Saccharomyces*, of which 78 strains were autochthonous fermentative yeasts. They were isolated from different niches (30 from grapes, 30 from leaves, 17 from the bark and 1 from the soil). According to the K1M detection test, 746 were commercial *S. cerevisiae* (K1M). The global distribution and frequency of fermentative yeasts population isolated from grapes, leaves, bark and soil, after spontaneous fermentation from the experimental vineyard over the 36 months studied are shown in Table 6.2.

**Table 6.2.** Global distribution and frequency of the commercial strains of *S. cerevisiae* (K1M) isolated after spontaneous fermentation from the experimental vineyard over the 36 months studied.

| | Control | Time 0 | 1 Week | 1 Month | 3 Months | 6 Months | 8 Months | 12 Months | 18 Months | 24 Months | 36 Months | Total |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Grape | | | | | | | | | | | | |
| Samples | 3 | 3 | 3 | 3 | - | - | - | 3 | - | 11 | 11 | 37 |
| Spontaneous Fermentations | 3 | 3 | 3 | 3 | - | - | - | 2 | - | 3 | 6 | 23 |
| Isolates of non *Saccharomyces* | 90 | 5 | 18 | 47 | - | - | - | 40 | - | 55 | 180 | 435 |
| Isolates of *Saccharomyces* non K1M | 0 | 0 | 0 | 0 | - | - | - | 0 | - | 30 | 0 | 30 |
| Isolates of *Saccharomyces* K1M | 0 | 85 | 72 | 43 | - | - | - | 20 | - | 5 | 0 | 225 |
| % of K1M | 0 | 94 | 80 | 48 | - | - | - | 33 | - | 6 | 0 | 30 |
| Leaf | | | | | | | | | | | | |
| Samples | 3 | 3 | 3 | 3 | - | - | - | 3 | - | 11 | 11 | 37 |
| Spontaneous Fermentations | 0 | 3 | 2 | 3 | - | - | - | 2 | - | 0 | 1 | 11 |
| Isolates of non *Saccharomyces* | 0 | 60 | 60 | 8 | - | - | - | 30 | - | 0 | 30 | 188 |
| Isolates of *Saccharomyces* non K1M | 0 | 0 | 0 | 0 | - | - | - | 30 | - | 0 | 0 | 30 |
| Isolates of *Saccharomyces* K1M | 0 | 30 | 0 | 82 | - | - | - | 0 | - | 0 | 0 | 112 |
| % of K1M | 0 | 33 | 0 | 91 | - | - | - | 0 | - | 0 | 0 | 15 |
| Wood | | | | | | | | | | | | |
| Samples | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 11 | 11 | 49 |
| Spontaneous Fermentations | 0 | 2 | 2 | 3 | 1 | 3 | 2 | 1 | 1 | 0 | 3 | 18 |
| Isolates of non *Saccharomyces* | 0 | 60 | 60 | 60 | 30 | 0 | 30 | 30 | 0 | 0 | 90 | 360 |
| Isolates of *Saccharomyces* non K1M | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 17 | 0 | 0 | 17 |
| Isolates of *Saccharomyces* K1M | 0 | 0 | 0 | 30 | 0 | 90 | 30 | 0 | 13 | 0 | 0 | 163 |
| % of K1M | 0 | 0 | 0 | 33 | 0 | 100 | 50 | 0 | 43 | 0 | 0 | 22 |
| Soil | | | | | | | | | | | | |
| Samples | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 11 | 11 | 49 |
| Spontaneous Fermentations | 0 | 2 | 1 | 3 | 3 | 3 | 3 | 3 | 0 | 0 | 2 | 20 |
| Isolates of non *Saccharomyces* | 0 | 0 | 0 | 48 | 58 | 82 | 46 | 59 | 0 | 0 | 60 | 353 |
| Isolates of *Saccharomyces* non K1M | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 |
| Isolates of *Saccharomyces* K1M | 0 | 60 | 30 | 42 | 32 | 8 | 44 | 30 | 0 | 0 | 0 | 246 |
| % of K1M | 0 | 100 | 100 | 47 | 36 | 9 | 49 | 33 | 0 | 0 | 0 | 33 |

The dissemination of K1M strain was efficient, high populations of yeast were found at time 0 (on the same day as dissemination) in grapes, leaves and soil (94, 33 and 100% respectively). All isolates in bark corresponded to non commercial *S. cerevisiae*. After a week the K1M strain was only isolated in grapes and soil in very high percentages (80 and 100%). Absence of yeast in the bark could be explained by the fact that this part of the plant is dryer than others; furthermore water and nutrients are concentrated mainly in grapes. One month later, close to senescence, the distribution of commercial yeast in the sprayed area was well represented in all parts of the vines (48% on grapes, 91% on leaves, 33% in the bark and 47% in the soil around the stump). According to Valero et al. (2007), during the period of ripeness, higher quantities of nutrients are available for yeasts, this favours their proliferation. Moreover sugars on leaves are transferred to bark as reservoirs in cold periods. At 3, 6 and 10 months during the resting stages of plants, the soil showed the maximum level of yeasts and the K1M strain was well represented in the bark, although a decreasing tendency was perceived. Twelve months after the voluntary dissemination, the percentage of K1M strain in the grapes and soil (both 33%) was lower than the values observed in the first week. At 18 months (March) only the bark contained the sprayed yeast (43%). At 24 months, the K1M strain had not survived in the sprayed plants, but a residual population (6%) was found in grapes situated 50 metres east of the dissemination area (Table 3). In the last sampling, at 36 months, commercial yeasts were not found in the vineyard. The dispersion and the number of isolates of K1M according to the time are shown in Figure 6.2.

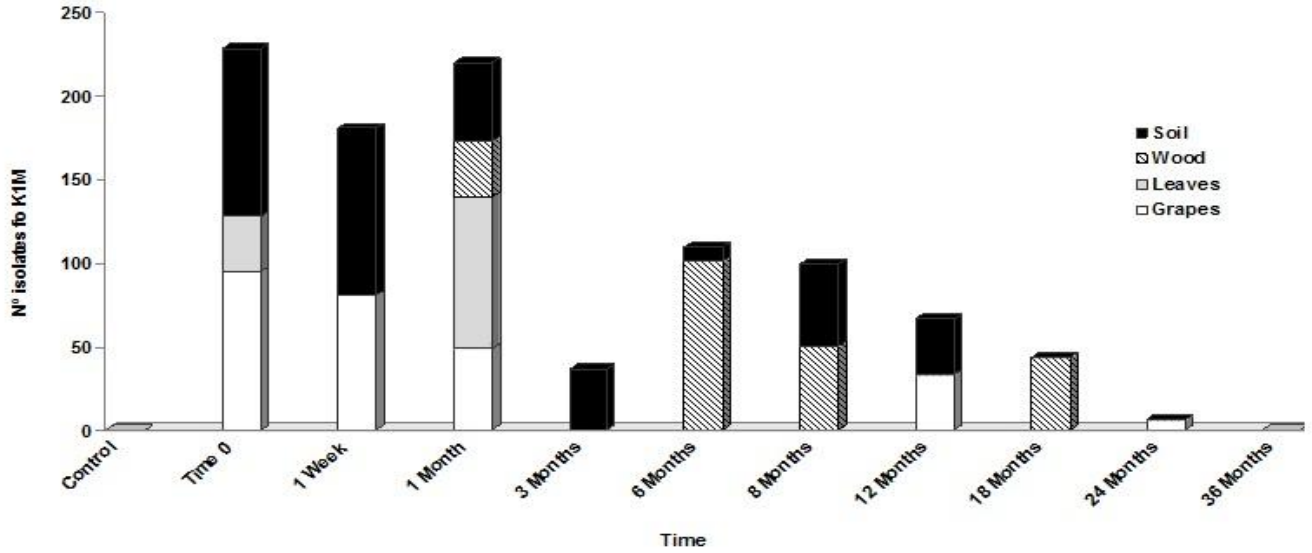



Figure 6.2. Overall distribution during three years of commercial yeast strain K1M

Microsatellite Multiplex PCR technique was used in order to confirm the results obtained. Colonies isolated from N+E+D medium were assayed to verify whether they had identical microsatellite patterns as commercial *S. cerevisiae* K1M (Lallemand), which was used as a control pattern. The 78 indigenous fermentative strains isolated from grapes, leaves, bark and soil were also analysed and compared with the profiles in the IMIDRA yeast collection database showing a unique profile different from K1M and belonging to native yeast of the Madrid winegrowing region (Table 6.4).

**Table 6.4.** Microsatellite patterns of *Saccharomyces cerevisiae* strains isolated in the disseminated vineyard (K1M* = commercial yeast pattern as control; K1M = disseminated commercial yeast; A = indigenous yeast).

| Genotype | Allele size (bp) | | | | | |
|---|---|---|---|---|---|---|
| | SCPTSY7-1 | SCPTSY7-2 | SC8132X-1 | SC8132X-2 | YOR267C-1 | YOR267C-2 |
| K1M* | 286 | 286 | 193 | 193 | 389 | 389 |
| K1M | 286 | 286 | 193 | 193 | 389 | 389 |
| A | 269 | 269 | 193 | 193 | 421 | 421 |

## 6.4. Discussion

Winemakers have used *S. cerevisiae* to make alcoholic beverages for thousands of years. Nowadays, this super-model research organism is central to advances in our biological understanding (Goddard et al. 2010). Fermentative yeasts populations comprise distinct domesticated and natural groups as well as mosaic strains, but a deep knowledge of the colonisation capacities and environmental risks of commercial yeasts is needed. Wineries are open systems and commercial yeasts have been used without any special control and could therefore be dispersed into the environment in large quantities via the sewage.

The importance of the colonisation and persistence of commercial strains of *S. cerevisiae* on the vineyard has also been highlighted in several studies (Comitini and Ciani, 2006; Schuller et al. 2005; Valero et al. 2005, 2007; Francesca et al. 2010). Nevertheless, there is no data available regarding the length of permanence of these yeasts in the vineyard or their presence in other vineyard niches. For this reason this study, carried out in the Madrid winegrowing region, includes aspects that have not been considered in previous works, such as the long-term survival of disseminated commercial yeast in grapes and must, or their permanence in different parts of the vine (leaves and bark) and the soil around the vine, which may act as the natural reservoir of these yeasts during the resting stages of the plants. In this way it is possible to obtain a more complete vision of the impact of commercial yeast on the natural microbiota of the vineyard.

In the present study 2160 strains were isolated, of which 1336 were non *Saccharomyces* yeasts, 78 strains were indigenous *S. cerevisiae* and, according to the K1M detection test, 746 were commercial *S. cerevisiae* (K1M), over a period of 36 months. Yeast biodiversity studies in the vineyard carried out by members of this research team in the Languedoc winegrowing region in the south of France (Valero et al. 2007) indicate that the methodology used showed an acceptable reflection of the initial biodiversity. This previous study showed a large proportion of non-*Saccharomyces* yeast species (66%), on the same level as our present study where these strains represented 63% of the total yeasts isolated over the 36 months. This new data confirms previous reports indicating that *S. cerevisiae* is not present in vineyards in large numbers (Pretorius, 2000).

Several authors considered that the origin of wine yeast is still controversial (Mortimer and Polsinelli, 1999; Pretorius 2000; Martini, 2003) in our study, a total of 78 strains of autochthonous *S. cerevisiae* were isolated. These results clearly indicate that indigenous fermentative yeasts occur in a very low percentage (4%) in vineyard ecosystems belonging to the Madrid winegrowing region. In their natural environment, yeasts have to cope with changing temperature, humidity (excess of water or drying), the effects of various toxic compounds coming either from the environment (e.g. drugs) or produced by other organisms in their immediate surroundings (Palková and Váchová, 2006). Spontaneous fermentations were achieved in order to obtain a significant population of these yeasts, owing to lower availability in the vineyard and with the aim of ascertain whether *S. cerevisiae* (K1M) was present in each sample. These samples yielded 78 colonies identified as indigenous *S. cerevisiae,* among which we found a unique genotype different from the K1M strain genotype (Table 4). We believe this provides strong evidence for a discrete population of fermentative yeasts residing in the sampled vineyard. Overall these communities were found on the grape berries and leaves (30 isolated in both niches) and 17 strains in bark. From soil only one strain was isolated, but it was the same genotype as the rest found in the different niches. This therefore appears to indicate that the native yeast found could be a possible “terroir” yeast candidate. In this way, by preserving and encouraging the autochthonous microbiota, their expression in wines could be asserted (Renouf et al. 2005; Francesca et al. 2010).

Regarding disseminated K1M, 34% of the total number of isolates from grapes, leaves, bark and soil, was the commercial yeast in the proposed sampling plan during 2006, 2007, 2008 and 2009. The highest proportion was found during the 2006 harvest, while a notable decrease

was perceived in 2007. In 2008, a residual population was found in grapes situated 50 metres east of the dissemination area, many factors could have influenced their transportation, such as geography, rainfall and wind direction (this year was the wettest, under low pressures the predominant winds usually blow from south-west (SW) to north-east (NE) in the vineyard area), birds, insects, small mammals and human-aided vectors. After 36 months, in 2009, no disseminated yeasts were found in the epicentre of the vineyard and within a radius of 100 metres around the disseminated area. This distance was determined by Valero et al. (2005) as the perimeter in which the dissemination is at a maximum.

Regarding the presence of K1M strains in the different niches during the 36 months, grapes and soil were their main reservoirs containing 30 and 33% respectively, of the total of the isolates. The other 22% was found in the vine bark and 15% of the commercial yeast corresponded to leaves. With this, soil and bark were the potential containers of these yeasts during the latency period given that they showed the highest values during the resting stages of the plants.

*Sensu strictu* species of the genus *Saccharomyces*, as their scientific name implies, usually need high concentrations of sugar and humidity during their biological cycle (Fay and Benavides, 2005). Nevertheless, grapes are sugar-rich environments related to the ripeness having a seasonal occurrence and consequently constitute one of the yeasts' habitats. K1M strains were sprayed over the vine (including grape berries) during the grape development. In this season higher sugar concentrations are present in grapes, making K1M yeasts' survival possible.

With respect to the soil, a high percentage of isolates of the K1M commercial yeast was found. This could be due to the vine building up organic matter by means of the leaves and old grapes during senescence periods. Thus, yeasts are provided with humidity and sugar and carbon sources. Moreover, in some yeasts of the genus *Saccharomyces*, sexual reproduction is triggered by adverse environmental conditions. A starved diploid cell enters meiosis and produces resistant haploid spores. This form of sexual reproduction however, usually results in the maintenance of the yeasts during the resting periods or very dry seasons.

According to Barnett (2000), *Saccharomyces* species exhibit a strong preference for hexoses such as glucose, fructose, mannose and galactose or simple oligosaccharides, specially maltose, sucrose and raffinose. In 2008, Sampaio and Gonçalves, analysed via HPLC the presence of these sugars in many tree bark samples from which fermentative yeasts were isolated. It was possible to detect the presence of at least one of the sugars in the bark. However, in the vine before senescence, high concentrations of sugar are transported from the leaves to the bark in order to have a nutrient reservoir during resting stages. Our results showed a high percentage of isolates of the disseminated *Saccharomyces* K1M between the third month and the eighth month of the study, when grapes and leaves were not present. This result suggests the availability of hexoses on the vine bark, where there was yeast growth.

The occurrence, and especially domination, of *Saccharomyces* in soils, plant leaves and decaying plant debris is extremely rare. The amount of exudates available to epiphytic microorganisms depends on a number of factors, such as cuticle thickness, the number of stoma, other anatomical features of the leaves (the presence of trichomes, glandular

fuzz, and extrafloral nectaries), which determine the availability of water and nutrients for epiphytic yeasts, which vary in plants of different ecological and taxonomic groups and necessarily affect yeast numbers (Glushakova et al. 2007). In earlier studies, epiphytic yeasts were counted mainly in the autumn, when their population and species diversity were maximal (Bab'eva et al. 1995; Glushakova and Chernov, 2007) or, rarely, three or four times for year (Inácio et al. 2002). The data obtained by Glushakova et al. (2007) indicates that *Saccharomyces* yeasts, contrary to the existing ideas about their predilection for sugar-rich substrates, are typical epiphytic species and can form a major part of the plant-associated yeast population.

Sláviková et al. (2007) demonstrated that yeasts were isolated from leaf surfaces of five species of fruit trees located in southwest Slovakia. Leaves are exposed to rapidly fluctuating temperature and relative humidity, which may have an impact on the yeast population. Large fluxes of UV radiation are also one of the most prominent features of the leaf surface environment to which microorganisms have presumably had to adapt (Lindow and Brandl 2003; Sláviková et al. 2007). To our knowledge, this is the first time that yeasts have been isolated from *Vitis vinifera* L. leaves in Madrid winegrowing region. A total of 188 non-*Saccharomyces* strains were isolated over the 36 months. The highest proportion was found at times 0 and 1 week, a significant decrease was observed after 1 month, nevertheless after 12 and 36 months a yeast population increase was observed. After 24 months, no yeasts were isolated. Indigenous *Saccharomyces* were also found at 12 months in vine leaves. Regarding disseminated commercial yeasts K1M, 30 and 82 strains were collected only at times 0 and 1 month owing to an initial presence. However, no survival was found in subsequent samplings.

Summarizing, our results show that permanent establishment of commercial yeast (K1M) in the vineyard did not occur and is restricted to short distances and a limited period of time. This result reinforce our previous findings (Valero et al., 2005, 2007), which showed that other factors were more important than commercial yeast utilisation for the biodiversity of the vineyard. Furthermore, no implantation was produced in the fermentation, as the presence of indigenous strains was not subsequently affected, principally after the first two years. It would appear that the environmental risks of the use of commercial yeasts strains were very limited. Our study demonstrates that commercial yeast (K1M) does not displace autochthonous *Saccharomyces*, and they are not sufficiently capable of colonisation and adaptation to new vineyard environments on a permanent basis. Nevertheless more long-term sampling could be advisable in order to confirm these results and the presence of commercial yeast must be monitored and, as far as possible, wineries must preserve the massive release of commercial yeasts in the environment in order to preserve the biodiversity of autochthonous microbiota.

# Capítulo 7

# DISCUSIÓN GENERAL

# Capítulo 7
# 7. Discusión general

***"Ciencia es todo aquello sobre lo cual siempre cabe discusión"***

- *Cita célebre del filósofo y ensayista español, José Ortega y Gasset.*

En el presente estudio se propuso como objetivo fundamental conocer y evaluar el efecto de distintos factores agronómicos relacionados con el manejo del viñedo sobre la microbiota de levaduras, principalmente fermentativas, asociadas a la uva, ya que son éstas las de mayor interés biotecnológico. De igual forma se pretendió evaluar la influencia de algunas prácticas enológicas, como la utilización masiva de levadura seca activa en la elaboración de vinos, y su posterior diseminación. Para ello se evaluó la capacidad de una levadura comercial, no autóctona de la zona en estudio, de colonizar y permanecer en diferentes nichos ecológicos del viñedo. Así, este capítulo trata de relacionar y dar una visión de conjunto de los resultados obtenidos a lo largo de los capítulos anteriores y que esto nos permita proporcionar información práctica, así como mostrar una serie de recomendaciones de utilidad para el sector vitivinícola.

Para la realización del estudio de los parámetros agronómicos (reflejados en los capítulos 3, 4 y 5), se fijaron 42 puntos de muestreo por campaña, recogiéndose un total de 126 muestras de uva a lo largo de los tres años del estudio. La recolección se realizó en el momento de la vendimia, cuando presentaron un nivel de madurez óptima para la elaboración de vinos. Los mostos obtenidos se fermentaron de forma espontánea en condiciones controladas de temperatura, agitación y volumen. Del total de 126 mostos obtenidos de las muestras de uvas,

solamente 86 desarrollaron la fermentación espontánea, llegando al menos a los 2/3 de la fermentación, cuando se habían desprendido 70 g/L de $CO_2$. En este punto se tomaron muestras de todos los mostos que llegaron a esta etapa de la fermentación, para el estudio de la flora fermentativa. De cada muestra se aislaron 30 colonias al azar. Se obtuvieron un total de 2580 aislados de los cuales, tras su identificación molecular, 446 pertenecieron al género *Saccharomyces* y 2134 a géneros no *Saccharomyces*. Todos ellos se agruparon en un total de 11 especies: *Candida apícola*, *Candida sorbosa*, *Candida stellata*, *Hanseniaspora guilliermondii*, *Kluyveromyces thermotolerans, Metschnikowia pulche-rrima*, *Pichia anomala*, *Pichia guilliermondii*, *Pichia toletana*, *Saccha-romyces cerevisiae* y *Torulaspora delbrueckii*. Las especies *K. thermo-tolerans*, *P. anomala* y *S. cerevisiae* fueron las más abundantes, por ese orden, en todos los estudios llevados a cabo en los capítulos 3, 4 y 5. Las levaduras de la especie *Saccharomyces cerevisiae* aisladas se identificaron a nivel de cepa, agrupándose en 10 genotipos diferentes. Dichas cepas de levaduras autóctonas, presentes en los viñedos de la Comunidad de Madrid, pueden presentar un importante recurso biotecnológico para la elaboración de vinos, principalmente en la D.O. “Vinos de Madrid”.

Paralelamente, se trató de esclarecer si una posible diseminación en el viñedo derivada del uso de levaduras comerciales en las bodegas próximas a éste, puede tener incidencia sobre el ambiente y sobre las poblaciones autóctonas de levaduras de interés para la industria enológica (capitulo 6). De igual forma, se trata de relacionar éstas prácticas así como las de los capítulos anteriores, desde el punto de vista de la conservación de la biodiversidad de la microbiota de levaduras presentes en el viñedo.

Como ya se ha indicado anteriormente, el objetivo de esta memoria es intentar determinar las condiciones de cultivo del viñedo más adecuadas, desde el punto de vista que permitan preservar y mejorar el la biodiversidad de las levaduras de interés enológico en la viña. Este trabajo está planteado sobre la realidad actual del viñedo siendo los diferentes parámetros ensayados perfectamente aplicables, si se adaptan a las necesidades del mismo.

## 7.1. El diseño experimental

Es importante destacar que los resultados obtenidos en los diferentes estudios de la influencia de los parámetros agronómicos sobre la microbiota de levaduras asociadas a la uva se han de tomar con cautela, ya que en los experimentos de campo existen siempre factores que son difíciles de controlar de un año a otro, como la climatología, radiación solar, velocidad del viento, etc. Siendo así, hemos intentado minimizar su influencia realizando los muestreos para el estudio de los distintos parámetros agronómicos en el mismo año de forma que se puedan comparar también entre sí, y repitiendo el estudio durante 3 campañas sucesivas. Todas las parcelas de los estudios correspondientes a los parámetros agronómicos (capítulos 3, 4 y 5) fueron, por tanto, muestreadas al mismo tiempo, en la misma localización geográfica, con el mismo tipo de suelo, así como otros factores referentes a la climatología, tipo de riego, etc, ya comentados en los capítulos anteriores. Por ello, no fue posible realizar un muestreo más amplio para cada una de las condiciones analizadas, ya que existía la limitación del número de muestras que podían ser manejadas al mismo tiempo. No obstante, como ya hemos mencionado los muestreos se realizaron durante tres campañas (2006, 2007 y 2008) y el número de muestras analizadas consideramos que puede ser suficiente para dar

una primera aproximación acerca de cómo los distintos parámetros agronómicos del viñedo influyen sobre las levaduras asociadas a la uva.

De igual modo, el propio diseño experimental elegido, utilizando la fermentación como método de enriquecimiento para la detección de levaduras fermentativas, no es un reflejo fiel de las poblaciones de levaduras presentes en la uva de forma natural. Nuestro interés en utilizar este método ha sido la detección preferente de levaduras con capacidad fermentativa del género *Saccharomyces,* que tienen una presencia escasa en las uvas sanas del viñedo (Fleet y Heard, 1993; Mortimer y Polsinelli, 1999; Valero et al., 2007). Dicho diseño experimental ha sido utilizado con éxito previamente por miembros de nuestro equipo de investigación (Schuller et al., 2005; Valero et al., 2005, 2007).

Debido a nuestra elección de realizar fermentaciones con los mostos procedentes de las uvas recolectadas, se prestó especial atención a la calidad del mosto obtenido para que pudiera llevarse a cado la fermentación espontánea de los mismos. Para ello se midió el contenido en azúcares y el pH en cada uno de los mostos, siendo éstos adecuados para el desarrollo de las fermentaciones. En ningún momento fue necesario un ajuste adicional del pH, dado que siempre se encontró entre los valores aceptables para que la fermentación se desarrollara sin inconveniente. Durante las fermentaciones, el mosto se mantuvo en agitación con objeto de mantener unas condiciones homogéneas, no obstante los fermentadores permanecieron tapados con un capilar que permitía la salida del $CO_2$ originado durante la fermentación, pero impedía la entrada de aire. Para favorecer el crecimiento de levaduras fermentativas, los aislamientos se realizaron en etapas avanzadas de la fermentación. Curiosamente en dichos aislamientos, realizados a los 2/3

de la fermentación, se aislaron además de levaduras del género *Saccharomyces*, algunas especies de levaduras perteneciente a distintos géneros no-*Saccharomyces*, como *K. thermotolerans*, *C. sorbosa* y *P. anomala* que pueden resultar también de gran interés para la industria del vino ya que muestran una elevada capacidad fermentativa. Algunos autores han mostrado que especies como *H. guilliermondii*, *K. thermotolerans*, *C. stellata*, *C. apicola* y *T. delbrueckii* podrían tener una mayor habilidad de crecer y permanecer hasta el final de la fermentación que *S. cerevisiae*, cuando la concentración inicial de azúcar es superior a 200 g/L (Benda, 1982; Lafon-Lafourcade, 1983; Kapsopoulou et al., 2007; Barrajón et al., 2009; Tofalo et al., 2009).

La misma razón de aislar preferentemente levaduras fermentativas, nos llevó a utilizar este sistema de muestreo para el estudio de la presencia en el viñedo de la levadura comercial K1M. Con este sistema tenemos la seguridad de que la presencia real de K1M en el viñedo va a estar siempre por debajo de la detectada tras la fermentación, dándonos un margen de seguridad a la hora de afirmar la presencia o no de la levadura comercial en la muestra analizada.

## 7.2. ¿Existe una práctica del viñedo ideal para preservar y favorecer las poblaciones de levaduras fermentativas asociadas a la uva?

Una cuestión como esta es siempre difícil de responder desde un único punto de vista ya que se pueden justificar opiniones de diversa índole. La “práctica ideal” como tal no existe, siempre está sujeta a consideraciones muy diversas como la influencia del medio natural en el que se cultiva la vid, las características geográficas, la variedad de la vid

propiamente dicha, el tipo de suelo, incluso el estilo de vino a elaborar y/o la rentabilidad deseada por parte de la bodega o empresa destinada a explotar un viñedo. Con estas condiciones, se podrían citar algunas de las claves para diseñar una práctica agrícola y enológica capaz de mantener, o al menos no desfavorecer, la presencia de levaduras autóctonas en la uva, capaces de realizar adecuadamente la fermentación. Además, probablemente su presencia podría contribuir a dotar al vino final de unas características organolépticas específicas. No se trata de evocar el cuestionado término *"terroir"*, pero sí de sugerir una serie de recomendaciones basadas en nuestros estudios para preservar las poblaciones de levaduras autóctonas y así no limitarnos los recursos biotecnológicos disponibles.

Aún no se ha demostrado que un producto, entre ellos el vino, obtenido a partir de la agricultura ecológica sea de mayor calidad que uno procedente de la convencional como corroboran numerosos estudios (Woese et al. 1997; Baker et al. 2002; Benbrook, 2005; Tarozzi et al. 2006). No obstante, en lo referente a la biodiversidad de levaduras autóctonas asociadas al viñedo, algunos autores (Hole et al. 2005) y nuestros resultados muestran que dicha biodiversidad se ve afectada negativamente por el uso de fitosanitarios empleados en la agricultura convencional.

Cuando se comparó la biodiversidad de un viñedo ecológico frente a un viñedo convencional, mediante la aplicación de los índices de biodiversidad de Shannon-Wiener (*H*'), Simpson (*D*) y la riqueza de especies (R) de levaduras en general (*Saccharomyces* y no *Saccha-romyces*), los resultados mostraron que entre ambos viñedos las diferencias no son significativas, a pesar de que el número de especies encontradas en el viñedo convencional fue menor, 5 frente a las 7 que

fueron obtenidas en el ecológico. Pero teniendo en cuenta el número de genotipos diferentes de las *S. cerevisiae* identificadas, el viñedo ecológico presentó una mayor biodiversidad que el convencional (9 frente a 1), este dato es de especial interés ya que son las levaduras fermentativas, capaces de finalizar la fermentación y por tanto de un mayor interés biotecnológico. Esto supone además una ventaja adicional para aquellos productores que basan la elaboración del vino en la fermentación espontánea. La conclusión principal que se desprende de los resultados es que los tratamientos fitosanitarios aplicados en el viñedo convencional, afectan negativamente a la microbiota de levaduras asociadas a la uva, especialmente a levaduras del género *Saccharomyces*.

Contrariamente a la ventaja que podría suponer el cultivo del viñedo de forma ecológica, en el estudio realizado sobre el tipo de manejo del suelo más favorable, resultó que el herbicida utilizado para mantener el suelo desnudo (glifosato) no tiene efectos negativos sobre la microbiota de levaduras de la uva, observándose incluso un notable incremento de la biodiversidad de levaduras en general y en la propia variabilidad de genotipos de *Saccharomyces cerevisiae*. De hecho, el número de genotipos encontrados en el viñedo así tratado fue mayor que en las otras dos estrategias estudiadas (suelo desnudo por laboreo y suelo con cubierta vegetal). Son numerosos estudios en los que se ha demostrado que las poblaciones de hongos, especialmente actinomicetos (como lo son algunas levaduras), bacterias y otros microorganismos se benefician del aporte de nutrientes que proporcionan algunos herbicidas (Roslycky, 1982; Araújo et al., 2003; Krzysko-Lupicka y Sudol, 2008), como también ocurre en el estudio que se presenta en esta memoria. Este hecho pudo estar ocasionado, según algunos autores (Haney et al., 2000; Johal y Huber, 2008), por el aporte de substratos energéticos por parte del

compuesto químico, siendo éstos aprovechados por los microorganismos tanto del suelo como de la uva, para su desarrollo. Sin embargo, hay que tener en cuenta que únicamente se analizó el efecto del glifosato como herbicida, lo que haría necesario la realización de más estudios con otros compuestos, bien de forma individual o en combinación, para corroborar estos resultados. Una alternativa al uso de herbicidas en lo que respecta al mantenimiento de una alta biodiversidad de levaduras en el viñedo, que además sería compatible con la agricultura ecológica, sería el suelo desnudo por laboreo. Esta opción podría ser interesante en climas áridos como lo es la región de Madrid, ya que el efecto del principio activo de los herbicidas en campo abierto se atenúa debido a las diferentes condiciones climáticas, especialmente las altas temperaturas y la lluvia (Roslycky, 1982; Haney et al., 2000; Busse et al., 2001; Johal y Huber, 2008). Este sistema es el más extendido entre los viticultores en cuanto a manejo del suelo, pero este tipo de práctica a la larga confiere un empobrecimiento y pérdida del suelo mediante la precipitación de partículas, erosión y un aumento en el riesgo de escorrentía, tal y como exponen Pastor et al. (2001). Una alternativa a este sistema y a la aplicación de herbicidas es el uso de las cubiertas vegetales, bien de carácter espontáneo o mediante el uso de especies diferentes de gramíneas o leguminosas. Dichas cubiertas vegetales aportan beneficios al suelo, como un incremento en la cantidad de materia orgánica y nutrientes, estructura y estabilidad en los agregados y mayor capacidad de retención del agua (Frye y Blevins, 1989; Aballay e Isunza, 2002; Tesic et al., 2007). Sin embargo, en regiones áridas y secas como es la cuenca del Mediterráneo, las cubiertas vegetales no son tan ventajosas, ya que requieren un mantenimiento mediante riego extra y pueden competir por el agua con la propia vid causando una disminución en el rendimiento de la misma (Pastor et al., 2001; White, 2009; Marques et al., 2010). Todos estos estudios han tenido como objetivo la vid, pero

ninguno ha observado la influencia del manejo de un suelo sobre las levaduras asociada a ella. Por primera vez, en esta memoria, se proporcionan datos al respecto. En nuestros resultados el manejo del suelo desnudo por laboreo puede ser la mejor alternativa aplicable en general a todos los tipos de viñedo para el mantenimiento de una biodiversidad de levaduras más elevada. El uso de cubiertas vegetales, no aportó ningún beneficio en cuanto a diversidad y cantidad de especies de levaduras, sino más bien al contrario.

Uno de los principales factores que se tienen en cuenta a la hora de adentrarse en el mundo del vino, es la elección de la variedad de uva que se va a utilizar para la elaboración del mismo. En la gran mayoría de los casos, esta variable está controlada por los distintos Consejos Reguladores de Denominaciones de Origen u organismos establecidos para el control de la misma en los diferentes países (Reglamento UE nº 401/2010 ; Real Decreto 1244/2008). Aunque como ya se ha comentado, su presencia es escasa en el viñedo, la uva puede ser una fuente natural de levaduras autóctonas fermentativas. Son numerosos los estudios sobre la composición y propiedades de las poblaciones de levaduras de la misma (Clemente-Jimenez et al. 2005; Raspor et al. 2006; Bauza et al. 2007; Pérez-Lamela et al. 2007; Chavan et al. 2009; Francesca et al. 2010), pero los estudios sobre cómo influye la propia variedad de uva sobre las levaduras asociadas a ella, son escasos. Wheeler y Crisp (2009) sugieren que uvas tintas derivadas de la agricultura ecológica dieron un producto final, mediante fermentación espontánea, de mayor calidad que vinos obtenidos de uvas tintas obtenidas de agricultura convencional, aunque no sucedió lo mismo en el caso de uvas blancas.

Las variedades de vid relativas a los estudios de la influencia de los parámetros agronómicos de esta Tesis: Garnacha, Syrah, Barbera y

Tempranillo, son conocidas por ser resistentes a enfermedades asociadas al viñedo y por su fácil crecimiento en climas secos y calurosos, como es la D.O. vinos de Madrid. La composición química y propiedades de la uva para la elaboración de vinos varían notablemente según la variedad de vid. Algunos de estos factores son el tamaño de la baya, el cuál influye directamente en la superficie de adhesión disponible para las levaduras (Renouf et al., 2005), la concentración de azúcares útiles para el desarrollo de las levaduras (Fleet, 2003), e incluso el grosor de la piel de la uva (Li et al., 2010). En el capítulo 3, se evaluó la influencia de la variedad de la vid sobre la biodiversidad de levaduras presentes en las uvas. Los resultados mostraron que el número de especies pertenecientes a géneros no *Saccharomyces* fue mayor que el de levaduras *Saccharomyces* en todas las variedades estudiadas, siendo la variedad Syrah la que más biodiversidad de estas especies albergó (8 especies de no *Saccharomyces* identificadas frente a las 4 encontradas en Garnacha y Barbera). Sin embargo, el contenido en levaduras del género *Saccharomyces* fue más representativo en la variedad Barbera, en la que se aislaron 112 cepas de *S. cerevisiae* entre las que se detectaron 5 genotipos diferentes siendo los genotipos B, D y F, exclusivos de esta variedad de vid. En Garnacha se encontraron 4 genotipos (E, G, H, I) también propios de la variedad. La variedad Syrah solamente presentó los genotipos A y C, los cuáles también se identificaron en Barbera. Renouf et al. (2005) muestran que cuando hay una gran disponibilidad en las uvas de superficie de adhesión para las levaduras y no se utilizan productos químicos para tratar el viñedo la microbiota asociada al mismo fue mayor. Nuestros resultados muestran que la variedad de vid influye sobre la distribución de poblaciones de levaduras autóctonas asociadas a la uva. Además es importante considerar que la mayoría de estos aislados se obtuvieron de mostos de uvas procedentes del viñedo ecológico. Por otra parte, cabe destacar

que algunas especies de no-*Saccharomyces,* como *P. toletana* y *C. sorbosa,* aparecen exclusivamente en la variedad Syrah, independientemente de los factores agronómicos, como se muestra en distintos capítulos de esta memoria. Esto podría ser una evidencia de una posible asociación variedad-cepa de levadura De acuerdo con Li et al. (2010), los factores varietales y el grosor de la piel de la uva influyen en la presencia de las distintas especies de levaduras.

En el estudio realizado del efecto de los fungicidas sobre las levaduras asociadas a la uva (capítulo 5), se realizó un muestreo sobre vides de la variedad Tempranillo, muy común y autóctona de España. Esta variedad también presentó una biodiversidad muy alta tanto de especies de géneros no *Saccharomyces* como de levaduras pertenecientes al género *Saccharomyces*. Se aislaron un total de 4 genotipos diferentes de levaduras de la especie *Saccharomyces cerevisiae* en la variedad Tempranillo, de los cuáles el genotipo denominado como D, fue exclusivo de esta variedad. El número total de genotipos identificados en las diferentes variedades de uva (Syrah, Garnacha, Barbera y Tempranillo) de los estudios relativos a los distintos parámetros agronómicos fue de 11. Estos resultados pueden ser de interés para el sector enológico, ya que la calidad final del vino no solamente está influenciada por las características varietales de la uva o la especie de levadura que lleve a cabo la fermentación, sino también por las cepas que participen en la misma (Pretorius, 2000; Callejón et al., 2010; Rodríguez et al., 2011), para lo cual es importante saber la riqueza de levaduras probable que aportaría cada cepa.

Otro objetivo que nos planteamos en este trabajo fue el de obtener más información sobre la influencia de los fungicidas en la diversidad y riqueza de levaduras fermentativas. Según esto, tenemos que

plantearnos ¿qué tipo de fungicida es más recomendable?, ¿en qué proporción?, ¿cuándo hay que aplicarlos?. Para dar respuesta a estas cuestiones se realizó un estudio del efecto sobre la biodiversidad de levaduras, de dos fungicidas (capítulo 5): el azufre (fungicida de amplio espectro) y el penconazol (fungicida sistémico contra el oídio), aplicados en 2 o en 4 estados fenológicos de la vid. Durante los tres años de estudio, además, hubo que tener en cuenta las variaciones climatológicas a la hora de aplicar los fungicidas, ya que en periodos más húmedos aumenta el riesgo de proliferación de hongos y la dosis necesaria es mayor. Nuestros resultados indican que los tratamientos fitosanitarios con fungicidas afectan negativamente a las poblaciones de levaduras. El tratamiento con azufre en dosis bajas (sólo dos aplicaciones en los estados fenológicos de desarrollo de la vid, floración y baya tamaño guisante) resultó ser el mejor ya que se encontró una mayor biodiversidad, especialmente de levaduras del género *Saccharomyces*. Es interesante destacar, que este tratamiento fue el utilizado en el viñedo ecológico analizado (capítulo 3) que presentó una alta biodiversidad en las poblaciones de levaduras, lo que corrobora este resultado.

Para el análisis global de los resultados parciales obtenidos en los diferentes ensayos y con la finalidad de comprobar si existe relación entre la distribución y frecuencia de las especies identificadas (Capítulos 3, 4 y 5) con los parámetros agronómicos analizados y llevar a cabo una clasificación de las mismas, se ha aplicado el análisis de funciones discriminantes (AFD) por pasos, utilizando como variables de clasificación los 14 parámetros analizados (viñedo convencional, viñedo ecológico, variedad Syrah, variedad Garnacha, variedad Barbera, variedad Tempranillo, suelo desnudo por laboreo, uso de herbicidas, cubierta vegetal, aplicación de azufre en dos estados fenológicos de la

vid, aplicación de azufre en cuatro estados fenológicos de la vid, aplicación de penconazol en dos estados fenológicos de la vid, aplicación de penconazol en cuatro estados fenológicos de la vid). Dos de las 10 funciones discriminantes canónicas obtenidas acumulan el 98,4% de la varianza explicada. Los coeficientes estandarizados de las variables para estas dos funciones discriminantes están recogidos en la tabla E.1. del apéndice E. Las variables Barbera, Garnacha, Syrah, Tempranillo, viñedo ecológico y suelo desnudo por laboreo, son las que presentan mayor poder discriminatorio. En la tabla E.2 del apéndice E aparecen los resultados de la clasificación de los especies de levaduras en función de las funciones canónicas obtenidas. Con estas funciones se consigue un porcentaje de aciertos en la clasificación de los aislados del 63,6%. Destacan las especies *K. thermotolerans*, *S. cerevisiae*, *P. anomala* y *Candida sorbosa* que han obtenido el 100% de aciertos en su clasificación.

En la representación gráfica de dicha clasificación (Figura E.1., apéndice E), se puede apreciar que la función 1 es la que más influye en la diferenciación entre las especies *K. thermotolerans* y *S. cerevisiae*, y la función 2 en la separación de *P. anomala*. El resto de las cepas analizadas a excepción de *Candida sorbosa* no están asociadas claramente a ninguno de los factores agronómicos considerados en el estudio, si bien, se muestran más representadas por la función canónica 2. Las especies *K. thermotolerans* y *S. cerevisiae* están más ligados al viñedo ecológico y a las variedades Barbera, Garnacha y Syrah, además se ven favorecidas por el empleo de azufre a dosis bajas. Por otra parte, *P. anomala* está correlacionada con las variedades Syrah y Barbera y favorecida por el mantenimiento del suelo desnudo por laboreo y el tratamiento con penconazol en dosis bajas.

Los resultados obtenidos en el conjunto de los estudios, validan que la variedad Barbera, en cultivo ecológico del viñedo y suelo desnudo por laboreo, con la aplicación de azufre en dosis bajas contribuye a la conservación de la biodiversidad de levaduras, principalmente de *S. cerevisiae* en el viñedo de la D.O. “vinos de Madrid”. De esta forma se ratifica que los parámetros agronómicos de manejo del viñedo tienen un papel importante en la biodiversidad de levaduras presentes en las uvas, lo que podría tener importantes repercusiones en el contexto vitivinícola.

## 7.3. ¿Es capaz una levadura comercial de implantarse en el viñedo?

Las levaduras comerciales son generalmente utilizadas en las bodegas sin ningún control especial. Las bodegas son sistemas abiertos y una vez finalizada la vinificación son eliminadas, en gran cantidad, junto con las aguas residuales y orujos procedentes de la vinificación, dispersándose en el medio ambiente. En numerosas ocasiones la bodega está situada en las proximidades del viñedo, por lo que las levaduras pueden llegar al mismo (Valero et al., 2005, 2007). Actualmente hay una gran falta de información en relación al comportamiento ecológico de estas levaduras en el viñedo. ¿Son capaces estas levaduras de sobrevivir en el ambiente pudiendo llegar a formar parte de la microbiota de la uva?, ¿pueden desplazar a la microbiota autóctona y participar en la fermentación de los mostos en años sucesivos a su utilización? ¿cuál sería su principal reservorio en el viñedo?. Para responder a estas y otras preguntas, se realizó una diseminación voluntaria en el viñedo, de una levadura comercial (K1M, Lalvin) con el objetivo de obtener datos fiables de su evolución y permanencia en la naturaleza (capítulo 6). Los resultados muestran que

la implantación permanente de la levadura comercial K1M en los viñedos de la Comunidad de Madrid no ocurre, quedando su presencia restringida prácticamente al primer año tras la diseminación y desapareciendo completamente al tercero. Además, no se produjo su implantación en la fermentación de los mostos de uvas sobre las que se ha diseminado la levadura, coexistiendo con las cepas indígenas. Según nuestros resultados, tras el primer año, los riesgos ambientales de la utilización de cepas de levaduras comerciales son muy limitados en el espacio y en el tiempo, no desplazando a la microbiota autóctona, estos resultados refuerzan los estudios llevados a cabo en Francia y Portugal (Valero et al., 2005, 2007). No obstante, aunque tenemos ya unas evidencias claras de que no hay una implantación permanente de la levadura comercial, sería conveniente la ampliación de estos trabajos con una mezcla de diferentes levaduras comerciales, principalmente debido a la importancia que podrían tener de cara a la evaluación de los riesgos ambientales de una posible utilización de levaduras genéticamente modificadas en la industria enológica.

# Capítulo 8

# CONCLUSIONES Y PERSPECTIVAS DE FUTURO

# Capítulo 8

# 8. Conclusiones y perspectivas de futuro

***"One might argue that the most important test tube in the birth and growth of the modern life sciences is the fermenter"***

- *From my colleagues Paul J. Chambers and I.Sakkie. Pretorius, (2010), "Fermenting Knowledge: the history of winemaking, science and yeast research", EMBO reports.*

## 8.1. Conclusiones

**Primera.-** Como era de esperar, la microbiota de levaduras presente en el viñedo fue mayoritariamente de géneros no–*Saccharomyces*. No obstante, la presencia y biodiversidad de *S. cerevisiae* encontrada, hace pensar en el viñedo como una fuente y potencial reservorio de levaduras de interés biotecnológico.

**Segunda.-** Los parámetros agronómicos de manejo del viñedo ejercen una influencia sobre la biodiversidad de las poblaciones autóctonas de levaduras asociadas a la uva.

**Tercera.-** El cultivo del viñedo según el sistema de agricultura ecológica presentó una mayor biodiversidad de especies de levaduras asociadas a la uva que cultivado de forma convencional.

**Cuarta.-** De las tres variedades de vid estudiadas, la variedad Barbera fue la que mostró una mayor biodiversidad de poblaciones de *Saccharomyces cerevisiae* asociadas a la uva, tanto en cultivo ecológico como convencional.

**Quinta.-** La práctica del suelo desnudo por laboreo resultó ser una opción sostenible respecto a la biodiversidad de levaduras en el viñedo. No obstante, el empleo de glifosato como herbicida no afectó negativamente a las poblaciones de levaduras, favoreciendo incluso el desarrollo de levaduras fermentativas.

**Sexta.-** El empleo de azufre en dosis bajas se muestra como un buen tratamiento antifúngico, desde el punto de vista de la biodiversidad y aplicable en cualquier sistema de producción agrícola. El empleo de penconazol redujo las poblaciones de levaduras asociadas a la uva en mayor medida que el azufre a cualquier dosis de aplicación, siendo más acusado este efecto sobre las poblaciones de *S. cerevisiae*.

**Séptima.-** La presencia de levaduras comerciales en el viñedo fue limitada en el espacio y en el tiempo. La cepa comercial K1M, no fue capaz de desplazar a las poblaciones autóctonas presentes en el viñedo y por tanto, el riesgo asociado de contaminación de los mostos en años sucesivos es muy débil.

**Octava.-** La uva y el suelo fueron los principales reservorios de la levadura comercial durante el periodo de envero y maduración de la uva, sin embargo durante la fase de reposo la mayor presencia de levaduras se encontró en el tronco.

## 8.2. Perspectivas de futuro

Normalmente un trabajo de investigación no suele ser algo cerrado, más bien al contrario, aporta ideas, diversifica y abre nuevas líneas de estudio que lo completen o resuelvan nuevas situaciones que se generen. Los resultados obtenidos en este trabajo nos han mostrado la

influencia que tienen los parámetros agronómicos sobre la riqueza de levaduras presentes en el viñedo, en nuestro estudio hemos abordado tres de estos factores y son muchos más los que podrían tener incluso una influencia mayor sobre las levaduras de la uva, tal sería el caso del riego o el sistema de conducción de la vid, etc que aún nos quedarían por estudiar.

El estudio de los fungicidas sobre la biodiversidad de levaduras presenta la novedad de estar realizado en campo, no obstante son muchos los ensayos "*in vitro*" que aún pueden hacerse para determinar realmente cual es el efecto de estos fungicidas sobre *S. cerevisiae* y sobre otras levaduras, y tal vez poder establecer algún programa de mejora de estas levaduras frente a ellos.

El tema de la cubierta vegetal que tanto se ha estudiado últimamente en el rendimiento del viñedo ha sido tratado por primera vez en el sentido de su posible influencia sobre las levaduras de la uva. De los resultados de esta Tesis se desprende que es mejor mantener el suelo desnudo, pero hay aún muchas cuestiones por responder ¿influyen por igual todos los tipos de cubierta vegetal? ¿habría una que fuera compatible con el mantenimiento de la riqueza de las poblaciones de levaduras?. Igual pasaría con las variedades de vid, se podrían estudiar las levaduras asociadas a cada una de las mismas y quizás tuviéramos la posibilidad de hacer análisis predictivos de la microbiota asociada a las mismas.

Tampoco podemos olvidar que en esta memoria nosotros nos hemos centrado en la obtención preferente de la microbiota fermentativa, por ello hemos incluido la fermentación en el diseño experimental, pero desde un punto de vista puramente ecológico también sería muy

interesante el aislamiento de la microbiota directamente de la uva y comparar estos resultados.

En cuanto al impacto ambiental de las levaduras comerciales en el ambiente, y su posible extrapolación al que tendrían levaduras genéticamente modificadas derivadas de ellas, aun queda mucho por hacer, por ejemplo diseminar voluntariamente varias levaduras a la vez, estudiar fenómenos de competencia y ver su evolución, como ocurriría en la realidad ya que difícilmente en una bodega se usa una única levadura comercial de forma continua.

Como hemos comentado al principio, esta nueva línea de investigación que pretende evaluar la influencia de los parámetros agronómicos del viñedo y algunas prácticas enológicas sobre la biodiversidad de levaduras de la uva, iniciada con el trabajo presentado en esta Tesis, no ha hecho nada más que empezar. Como se muestra en los resultados, son parámetros a tener en cuenta tanto por el viticultor como por el enólogo, con el objetivo conjunto de mejorar los procesos de producción del vino y la calidad del producto final, respetando y aprovechando los recursos microbianos o biotecnológicos presentes en cada zona vitivinícola.

# Apéndice A

# MATERIAL Y MÉTODOS

# Apéndice A

# A. Material y Métodos

## *A.1. Procesado de las muestras y aislamiento de levaduras*

En cada punto de muestreo se tomaron entre 1 y 2 Kg de uvas en condiciones asépticas en bolsas de congelación, posteriormente se transportaron en una nevera al laboratorio. Directamente en las bolsas se procedió al estrujado de las uvas y con el mosto resultante se llevaron a cabo los siguientes pasos:

- 80 ml de mosto se dispensaron en un fermentador.

- 100µl del mosto se extendieron (inoculó) en placa con medio YPD compuesto por extracto de levadura (1%), peptona de carne (1%), glucosa (2%) y en el caso de medio sólido se le añadió agar (2%). El medio fue esterilizado en autoclave a 121ºC durante 20 minutos, para realizar los aislamientos de la microbiota inicial.

- Aproximadamente 50 mL del mosto obtenido se centrifugaron a 14.000 rpm, durante 5 minutos a 4ºC, el sobrenadante se congeló a -40ºC para posteriores análisis químicos.

- El mosto restante se centrifugó durante 5 minutos a 5000 rpm, se tomó el sobrenadante y se midió el pH y el contenido en azúcares reductores por refractometría (refractómetro digital modelo ATAGO CO, LTD. Tokio, Japón) y el grado alcohólico probable del mosto por conversión del grado

Baumé mediante el empleo de tablas específicas autorizadas. El resto se conservó a -20ºC.

- Las fermentaciones se realizaron con 80 ml de mosto en fermentadores de 100 ml de capacidad tapados con un tapón con capilar a 20ºC con agitación de 150 rpm (Fig. A. 1). El seguimiento de la fermentación se realizó según la cantidad de $CO_2$ desprendido, calculando la diferencia entre la pesada diaria y el peso inicial del fermentador. A los 70 g/l de $CO_2$ desprendido (aproximadamente a los 2/3 de fermentación), se tomaron las muestras para el aislamiento de levaduras según la técnica de las diluciones sucesivas y siembra de 100µl de cada dilución en placa con medio YPD.


**Fig. A.1.** Imagen de los fermentadores de 100 ml de capacidad en los cuales se llevó a cabo la fermentación de cada una de las muestras.

Para el aislamiento se eligieron las placas cuyo nivel de dilución permitía obtener entre 30 y 300 UFC/ml. Se tomaron 30 colonias al azar de cada muestra, se incubaron a 28ºC en medio YPD y se conservaron por congelación a – 80ºC para su posterior identificación. La flora inicial decidimos no incluirla porque era casi imposible de aislar por la gran cantidad de hongos.

## A.2. Identificación de levaduras

La primera clasificación de las levaduras del capítulo 6 se realizó en base al test de crecimiento en L-lisina como única fuente de nitrógeno (Barnett et al. 2000), lo que conduce a la división de las cepas de levaduras aisladas en dos grandes grupos, *Saccharomyces* y no-*Saccharomyces*, en base a la incapacidad de las levaduras del género *Saccharomyces* a crecer con lisina como única fuente de carbono.

Para la realización del test son necesarios dos medios de cultivo, el primero llamado YNB Lys – , compuesto por YNB (Yeast Nitrogen Base) sin aminoácidos y con sulfato amónico 0,67%, glucosa 2%, agar 2% y el segundo medio con lisina como única fuente de nitrógeno YNB Lys + compuesto por YNB sin aminoácidos ni sulfato amónico 0,67%, glucosa 2%, agar 2% y lisina 0,2%. Ambos fueron esterilizados en autoclave a 121ºC durante 20 minutos.

Las levaduras que no presentaron crecimiento en medio con lisina como única fuente de nitrógeno se asociaron al género *Saccharomyces*, las que sí crecían en el medio con lisina se consideraron levaduras no Saccharomyces (Fig. A.2)

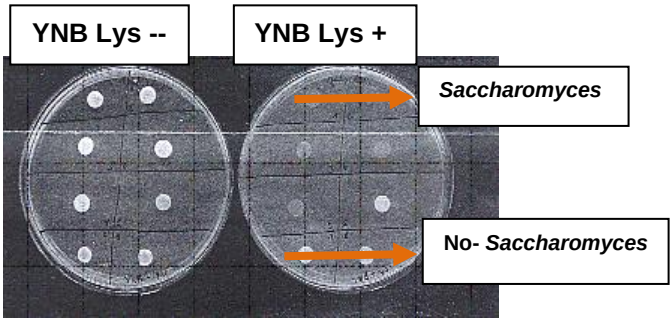



**Fig. A.2.** Test de la lisina. La placa con medio YNB Lys **–** muestra crecimiento de todas las levaduras, por el contrario en la placa con medio YNB Lys + se observa crecimiento de aquellas especies capaces de asimilar lisina como única fuente de nitrógeno.

# *A.3. Identificación genética de las cepas de levadura aisladas*

## *A.3.1. Extracción del ADN*

En la identificación genética de las levaduras, el primer paso necesario es la extracción del ADN. Para la obtención de la biomasa, la levadura a identificar se inoculó en 25 ml de medio YPD líquido y se incubaron a 28ºC, con agitación 128 rpm durante 24 - 48 horas (Minitron INFORS). Para la extracción del ADN se utilizó un kit comercial de extracción de ADN para levaduras (ArchivePure DNA Purification System) de la casa comercial 5 Prime (Eppendorff). Se utilizó el protocolo indicado modificado por nosotros según los siguientes pasos:

- Lisis celular

1. Añadir 1 ml de un cultivo de al menos 12 horas y que tenga de 1-2 x $10^8$ células a un Eppendorf de 1,5 ml. Mantener en hielo.
2. Centrifugar a 14.000 rpm durante 1 minuto, tirar el sobrenadante.
3. Añadir 300 µl de *Cell Suspensión Solution* y resuspender el pellet con una pipeta.
4. Añadir 1,5 µl de *Lytic Enzyme Solution*, invertir el tubo 25 veces para mezclarlo bien.
5. Incubar a 37º durante 30 minutos para digerir las células. Invertir el tubo de vez en cuando durante la incubación.
6. Centrifugar a 14.000 rpm durante 1 minuto. Tirar el sobrenadante.

7. Añadir 300 µl de *Cell Lysis Solution y* resuspender el pellet.

- Precipitación de proteínas

1. Añadir 100 µl de *Protein Precipitation Solution* al lisado de células.
2. Agitar en Vortex a la máxima velocidad durante 20 segundos.
3. Centrifugar a 14.000 rpm durante 3 minuto. Debe formarse un pellet, de no ser así, repetir el paso 2 seguido de una incubación en hielo de 5 minutos y luego repetir el paso 3.

- Precipitación del ADN

1. Recoger el sobrenadante con el ADN en un eppendorf de 1,5 ml que contenga 300 µl de Isopropanol al 100%.
2. Mezclar por inversión 50 veces.
3. Centrifugar a 14.000 durante 1 minuto, el ADN debería observarse como un pequeño pellet blanco.
4. Tirar el sobrenadante y secar el tubo con papel absorbente. Añadir 300 µl de etanol al 70% e invertir el tubo varias veces para lavar el pellet.
5. Centrifugar a 14.000 rpm durante 1 minuto. Tirar el etanol con mucho cuidado.
6. Invertir y secar el tubo con papel absorbente, secar en el Speed-Vac (Thermo electro corporation) de 5 a 10 minutos a 35ºC.

- Rehidratación del ADN y tratamiento con RNA-asa

1. Añadir 50 µl de *DNA Hydration Solution.*
2. Añadir 1.5 µl de *RNA-asa A Solution* para purificar la muestra.

3. Mezclar con vortex 1 segundo. Dar un *spin* para recoger todo el líquido e incubar a 37ºC durante 1 hora.
4. Para que el DNA se continúe hidratando, incubar otra hora a 65ºC o dejar toda la noche a temperatura ambiente.
5. Guardar las muestras a 4ºC. Para largos períodos de conservación congelar a -20 ºC ò -80ºC.

### *A.3.2. Cuantificación del ADN extraído e identificación molecular de levaduras*

Las técnicas de Biología Molecular utilizadas para la identificación molecular requieren un alto grado de pureza del ADN. Para la cuantificación del ADN se utilizó el espectrofotómetro UV-Vis NanoDrop 1000 (Thermo Fisher Scientific Inc.) que cubre el rango espectral de 220 a 750nm. Éste nos permitió obtener datos numéricos de la concentración del ADN extraído, siendo la ideal aquella comprendida entre 20-80 ng/µL
La identificación molecular de las levaduras aisladas se llevó a cabo a través de técnicas de Biología Molecular que han permitido hacer una clasificación más precisa, llegando a diferenciar cepas de una misma especie, como es el caso de *S.cerevisiae*. Este método se basa en la utilización de las técnicas que se describen a continuación:

### *A.3.3. Estudio de las secuencias de los espaciadores intergénicos: regiones ITS del ADNr mediante PCR*

La amplificación de la región ITS del ADN ribosómico se llevó a cabo añadiendo 1,5 µl del ADN objeto de estudio a 18,5 µl de una mezcla para PCR compuesta de los siguientes productos:

| Compuesto | Concentración del Stock | Concentración final | µL de reacción/20µL |
|---|---|---|---|
| **$MgCl_2$** | 25 mM | 2,5 mM | 1,6 |
| **dNTP** | 10 mM | 0,4 mM | 0,4 |
| **Buffer** | 10 X | 1 X | 2 |
| **Primers ITS1-ITS4** | 10 µM | 1 µM | 0,4 |
| **Taq-DNA polimerasa** | 5 U/µL | 0,125 U/µL | 0,2 |
| **Agua** | Pura | En volumen | 14,5 |
| **ADN** | 20-80 ng/µL | 20-80 ng por reacción | 1'5 |

Los *primers* utilizados fueron: ITS1 (5' TCCGTAGGTGAACCTGCGG 3') y ITS4 (5' TCCTCCGCTTATTGATATGC 3') suministrados por MWG Biotech AG (Ebersberg, Alemania). Las condiciones de la PCR fueron las siguientes: una desnaturalización inicial a 95ºC durante 5 minutos; 35 ciclos de desnaturalización a 95ºC durante un minuto, alineamiento a 55ºC durante 1 minuto, una extensión durante 1,5 minutos a 72ºC; y una extensión final a 72ºC durante 7 minutos. Todas las PCRs fueron realizadas en un termociclador MRW Biotech.

### *A.3.4. Análisis de los fragmentos de restricción del polimorfismo de longitud (RFLP)*

La determinación de los RFLPs se realizó sobre el producto de la PCR-ITS utilizando las endonucleasas de restricción *Hae*III, *Cfo*I e *Hinf*I (Promega Biotech) a 37ºC durante la noche. La mezcla para la digestión por dichas enzimas de restricción fue:

| Compuesto | µL de reacción |
|---|---|
| **Enzima (*Hinf* I, *Hae* III, *Cfo* I)** | 0.5 |
| **Buffer** | 1 |
| **Agua** | 3,5 |
| **ADN** | 5µL |

### *A.3.5. Análisis de amplificación al azar de ADN polimórfico (RAPD-PCR)*

La técnica de RAPD permite la amplificación de regiones anónimas de ADN mediante el empleo de *primers* arbitrarios. Para esta técnica es necesario que la concentración del ADN sea entre 20 – 80 ng/µL y la pureza del ADN es fundamental para el éxito del análisis. Se utilizó el *primer* OPB-15 MWG Biotech AG (Ebersberg, Alemania) que contiene la siguiente secuencia: 5'-GGAGGGTGTT-3'**.** Además se utilizaron los compuestos clásicos de la técnica PCR descritos a continuación:

| Compuesto | Concentración del Stock | Concentración final | µL de reacción/20µL |
|---|---|---|---|
| **$MgCl_2$** | 25 mM | 2,5 mM | 4 |
| **dNTP** | 10 mM | 0,4 mM | 0,8 |
| **Buffer** | 10 X | 1 X | 2 |
| **Primer OPB-15** | 10 µM | 1.0 µM | 2 |
| **Taq-DNA polimerasa** | 5 U/µL | 0,125 U/µL | 0,5 |
| **Agua** | Pura | En volumen | 9,7 |
| **ADN** | 20-80 ng/µL | 20-80 ng por reacción | 1 |

En cada tubo se alícuota 19µl de mezcla de reacción y 1µl de ADN que hacen un volumen final de 20µl. Las condiciones de la PCR fueron las siguientes: un precalentamiento inicial a 95ºC durante 4 minutos; 45

ciclos de 95ºC durante 1 minuto; 36ºC durante 1 minuto y 72ºC durante 2 minutos; un ciclo final a 72ºC durante 5 minutos.

### *A.3.6. Análisis mediante Microsatélites o secuencias simples repetidas (SSR) (Simple Sequences Repeats Multiplex)*

Se analizaron tres microsatélites en una reacción múltiple (multiplex): SC8132X, YOR267C y SCPTSY7, para los cuales se utilizaron los *primers* indicados en la tabla A.1, todos ellos de MGW Biotech AG (Ebersberg, Alemania), dichos microsatélites fueron elegidos por su alto grado de polimorfismo (Field y Wills, 1998; González Techera *et al*., 2001; Vaudano y García-Moruno, 2008).

Tabla A.1. Relación de *primers* utilizados en la técnica SSR multiplex.

| Locus | Cromosoma | Secuencia | Bibliografía |
|---|---|---|---|
| **SC8132X** | XVI | FW: CTGCTCAACTTGTGATGGGTTTTGG<br>RV: CCTCGTTACTATCGTCTTCATCTTGC | Field y Wills (1998). |
| **YOR267C** | XV | FW: GGTGACTCTAACGGCAGAGTGG<br>RV: GGATCTACTTGCAGTATACGGG | Field y Wills (1998).<br>González-Techera et al., (2001). |
| **SCPTSY7** | XIII | FW: AAAAGCGTAAGCAATGGTGTAGAT<br>RV: AAATGATGCCAATATTGAAAAGGT | Pérez et al., (2001).<br>Vaudano y García-Moruno (2008). |

La mezcla de reacción de la PCR multiplex fue la siguiente:

| Compuesto | Concentración del stock | Concentración final | µL en 20 de reacción |
|---|---|---|---|
| **MgCl2** | 25 mM | 3.1 mM | 2,5 |
| **dNTP** | 10 mM | 0,4 mM | 0,8 |
| **Buffer** | 10X | 1X | 2 |
| **Primer SCPTSY7** | 4 µM | 2 µM | 4 |
| **Primer SC8132X** | 1,5 µM | 0,75 µM | 1,5 |
| **Primer SCYOR267C** | 1 µM | 0,5 µM | 1 |
| **Taq-DNA polimerasa** | 5 U/µL | 0,125 U/µL | 0,4 |
| **ADN** | 20-80 ng/L | 25-100 ng por reacción | 1,5 |

Las condiciones de la PCR fueron las siguientes: precalentamiento inicial a 94ºC durante 4 minutos; 28 ciclos de: 94ºC durante 30 segundos, 56ºC durante 45 segundos, 72ºC durante 30 segundos; un ciclo final a 72ºC durante 10 minutos. (Vaudano y Garcia-Moruno, 2008).

## *A.4. Visualización de los perfiles genéticos*

La visualización de los perfiles genéticos se llevó a cabo por electroforesis en un gel de agarosa a una concentración de 1,4% en el caso de la PCR-ITS y RAPD-PCR y de 2,5% en el caso de los RFLP y SSR, diluída en un tampón TBE 1X (Tris/Borate/EDTA). A esta mezcla se le añadió 5µl/mL de Bromuro de Etidio como agente intercalante. Para las técnicas de PCR-ITS, RFLP y SSR se utilizó un marcador molecular de 100 pb ladder (Promega) y en el caso de la PCR-RAPD 1kb ladder (Promega).

El gel fue sometido a un campo eléctrico de 100 V durante 120 minutos. Finalizada la electroforesis se procedió a la visualización del gel en un transiluminador UV (Spectroline U.V. Transilluminator) y posteriormente al fotografiado del mismo con una cámara acoplada al sistema (Gel Logic 200 Imaging System, Kodak). Para el análisis de los tamaños de los fragmentos de restricción se utilizó el software Kodak

Molecular Image Software. Además, se comprobó el tamaño de los fragmentos y se determine el tamaño de los alelos de las diferentes cepas de S. cerevisiae mediante electroforesis capilar simple en un secuenciador automático ABI 3130 (Applied Biosystem).

## *A.5. Análisis genómico de las levaduras*

El diseño de los oligonucleótidos empleados en la amplificación por PCR se llevó cabo a través de la base de datos y el software de *Saccharomyces* Genome Database (SGD, genome-www.stanford.edu). El diseño de *primers* para el análisis RAPD y SSR se realizó con el apoyo del software Primer3 (http://frodo.wi.mit.edu/cgibin/ primer 37 primer3_www.cgi).

## *A.6. Análisis de los resultados*

Las levaduras fueron identificadas a nivel de especie mediante la comparación de los productos amplificados y el tamaño de los fragmentos de restricción descritos previamente por Guillamón et al. (1998), Esteve-Zarzoso et al. (1999) y Fernández-Espinar et al. (2000), para las especies control. Además en cada reacción de amplificación y restricción, se utilizaron como referencia cepas tipo procedentes de la Colección Española de Cultivos Tipos (CECT): *Torulaspora delbrueckii* CECT1015, *Pichia guilliermondii* CECT11029, *Metschnikowia pulcherrima* CECT10071, *Pichia toletana* CECT11493, *Pichia anomala* CECT1110, *Saccharomyces cerevisiae* CECT1176, *Hanseniaspora guilliermondii* CECT11029, *Kluyveromyces thermotolerans* CECT1962, *Candida sorbosa* CECT11204 and *Candida stellata* CECT11918 y *Candida apicola* CECT11167.

El control de los perfiles de en la RAPD-PCR y SSR se realizó mediante el análisis de los mismos y tomando como referencia las cepas tipo de la CECT. Para este estudio de la variabilidad genética de poblaciones autóctonas de levaduras de interés, se aplicaron diferentes métodos estadísticos con el programa SPSS v.16.0 y Bionumerics.

# Apéndice B

# ABREVIATURAS

## Apéndice B

# Abreviaturas

| | |
|---|---|
| a. C | antes de Cristo |
| ADN | ácido desoxirribonucleico |
| AP | amplified product |
| bp | base pair/pares de bases |
| C | Carbón/Carbono |
| ºC | grado centígrado/Celsius degree |
| cc | cubiques centimetres/centímetros cúbicos |
| CECT | Colección Española de Cultivos Tipo |
| cfu | colonies former units |
| Cl | Clore/Cloro |
| cm | centimetre/centímetro |
| Cu | Copper/Cobre |
| CV | conventional vineyard |
| *D* | Simpson Index/Índice de Simpson |
| d. C. | después de Cristo |
| DNA | deoxyribonucleic acid |
| DNase | deoxyribonuclease |
| dNTPs | 2'-deoxynucleosides 5'-triphosphate |
| D.O. | Denominación de Origen/Apellation of Origin |
| E | East/Este |
| E. C. | European Community |
| EDTA | ethylenediamine-tetraacetic acid |
| Fig. | Figure/figura |
| for | forward |
| g | grame |
| H | Hidrogen/Hidrógeno |
| *H'* | Shannon-Wiener index/índice de Shannon-Wiener |

| | |
|---|---|
| h | hour/hora |
| Ha | Hectarea |
| HL | Hectolitre/Hectolitro |
| ITS | Internal Transcription Space |
| Kb | Kilobase |
| Kg | Kilogrames/Kilogramo |
| L | Litre |
| L. | Linné/Linneo |
| Lys | Lysine/Lisina |
| M | molar/Mol |
| m | milimetros/millimetres |
| Mg | Magnesium/Magnesio |
| min | minute/minuto |
| mit | mitochondrial/mitochondrial |
| mL | millilitre/mililitro |
| mm | milimetro/millimetre |
| N | Nitrogen/Nitrógeno |
| N | North/Norte |
| NED | N medium with Erythromycin and Diuron |
| ng | nanograme |
| nm | nanometre |
| O | Oxigen/Oxígeno |
| OD | optical density |
| OV | organic vineyard |
| PCR | Polymerase Chain Reaction |
| rev | reverse |
| RAPD | Random Amplified Polymorphic Deoxyribonucleic |
| RFLP | Random Fragments Length Polymorphic |
| RNA | ribonucleic acid |
| rADN | ácido desoxiribonucleico ribosomal |

| | |
|---|---|
| rARN | ácido ribonucleic ribosomal |
| rRNA | ribosomal ribonucleacid |
| RNase | ribonuclease |
| rpm | revolutions per minute |
| S | Sulfur/Azufre |
| S | South/Sur |
| *S* | richness/riueza de species |
| s | second/segundo |
| S.D. | standard deviation |
| sp. | Species |
| SPSS | Statistical Product and Service Solution |
| SSR | Simple Sequence Repeats |
| TBE | Tris-Borate-EDTA |
| U | Units/Unidades |
| UV | ultraviolet/ultravioleta |
| V | volts/voltios |
| v/v | volume/volume-volumen/volumen |
| W | West/Oeste |
| w/v | weight/volume-peso/volume |
| X g | Gravity Unit |
| YNB | yeast nitrogen base |
| YPD | yeast peptone dextrose medium |

# Apéndice C

# FACTORES DE CONVERSIÓN DEL SISTEMA INTERNACIONAL DE MEDIDAS (S.I) Y NO INTERNACIONAL

| Conversión de columna 1 en columna 2, multiplicar por: | Columna 1 S.I. | Columna 2 no S.I. | Conversión de columna 2 en columna 1, multiplicar por: |
|---|---|---|---|
| **Longitud, área y volumen** | | | |
| 3,28 | metro (m) | pie (ft) | 0,304 |
| 39,4 | metro (m) | pulgada (in) | 0,0254 |
| $39,4 \times 10^{-2}$ | milímetro (mm) | pulgada (in) | 25,4 |
| 2,47 | hectárea (ha) | acre (ac) | 0,405 |
| 0,265 | litro (L) | galón | 3,78 |
| $9,73 \times 10^{-3}$ | metro cúbico ($m^3$) | acre-pulgada (ac-in) | 102,8 |
| $8,11 \times 10^{-4}$ | metro cúbico ($m^3$) | acre-pie (ac-ft) | $1,233 \times 10^{-3}$ |
| 35,3 | metro cúbico ($m^3$) | pie cúbico ($ft^3$) | $2,83 \times 10^{-2}$ |
| 0,811 | Megalitro (ML) | acre-pie (ac-ft) | 1,233 |
| **Masa** | | | |
| $2,20 \times 10^{-3}$ | gramo (g) | libra (lb) | 454 |
| 2,205 | kilogramo (kg) | libra (lb) | 0,454 |
| 1,102 | tonelada (t) | tonelada (U.S.A) (ton) | 0,907 |
| **Cantidad por unidad de área** | | | |
| 0,893 | kilogramo/hectárea (kg/ha) | libra/acre (lb/ac) | 1,12 |
| 0,446 | tonelada/hectárea (t/ha) | tonelada/acre (U.S.A) (ton/ac) | 2,24 |
| 0,107 | litro/hectárea (L/ha) | galón/acre | 9,35 |
| **Miscelánea** | | | |
| (9/5ºC) + 32 | Celsius (ºC) [a] | Fahrenheit (ºF) | 5/9 (ºF - 32) |
| 9,9 | megaPascales (MPa) | atmósferas | 0,101 |

[a] para convertir grados Celsius (ºC) a grados Kelvin (ºK), sumar 273.

[b] mega (M), $x10^6$; kilo (k), $x10^3$; deci (d), $x10^{-1}$; centi (c ) $x10^{-2}$; mili (m), $x10^{-3}$; micro (µ), $x10^{-6}$; nano (n), $x10^{-9}$.

[c] no existe conversión directa de revoluciones por minuto (rpm) a unidades de gravedad (X g) ya que depende del rotor, para facilitar la conversión hay disponible en la red numerosos conversores, www.currentprotocols.com/tools/g-forcerpm.conversion-tool

# Apéndice D

# VIÑEDO, SUELO Y CLIMA

# Apéndice D
# Viñedo, suelo y clima

## D.1. Viñedo

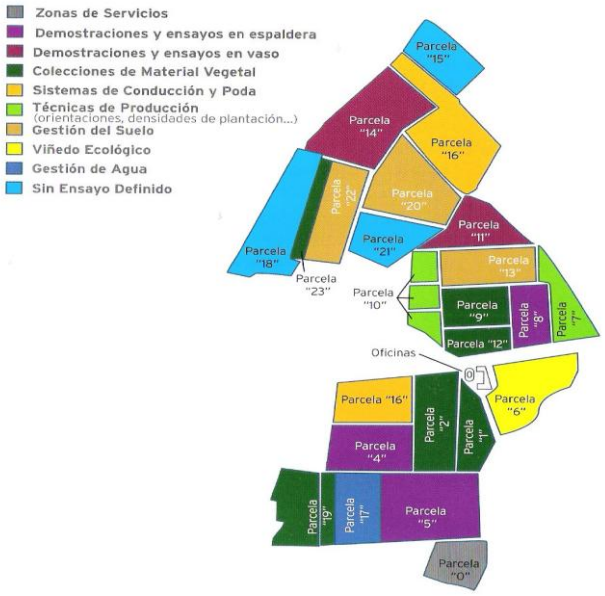
Parte de la experimentación y la recogida de muestras de los trabajos correspondientes a los capítulos 3, 4 y 5 se llevaron a cabo dentro de la Denominación de Origen “Vinos de Madrid” en el Centro de Transferencia Tecnológica Vitivinícola “El Socorro”. Por otro lado, el trabajo desarrollado a lo largo del capítulo 6, se realizó en un viñedo de la finca “El Encín”, también incluida en la citada denominación. En este anexo se detallan los aspectos más importantes de ambas áreas, como son el clima, suelo y las características más relevantes en cuanto a las vides formadoras de los viñedos se refiere.

### *D.1.1. C.T.T.V. “El Socorro”*

El C.T.T.V. “El Socorro” se puso en marcha en 1997 a través de un convenio de colaboración suscrito entre el actual Instituto Madrileño de Desarrollo Rural, Agrario y Alimentario (IMIDRA) y el grupo de viticultura del departamento de Producción Vegetal: Fitotecnia, de la Escuela Superior de Ingenieros Agrónomos de Madrid, con el objetivo de impulsar el desarrollo del Sector Vitivinícola.

Los viñedos de “El Socorro” se encuentran entre las localidades de Belmonte de Tajo, Colmenar de Oreja y Valdelaguna, en la intersección entre las carreteras M-404 y M-315 de la Comunidad de Madrid. Las coordenadas geográficas son 40° 8' 1.5864" N, 3° 22' 26.9754" W y 743 m altitud. La finca dispone de 23 hectáreas divididas en 23 parcelas de

carácter experimental, donde se reúnen los más diferentes viñedos y los distintos factores de la producción vitícola se encuentran representados en sus más diversas expresiones, de manera que satisfagan las necesidades de los viticultores, empresas, estudiantes e investigadores (Fig. D.1.). En este centro experimental se desarrollan actividades a partir de un conjunto de viñedos de carácter polivalente que evolucionan adaptándose en cada momento a las exigencias del sector. El centro cuenta con un equipo multidisciplinar de trabajo, integrado por profesionales con diverso grado de especialización y con los materiales necesarios para desarrollar las actividades y alcanzar los objetivos propuestos. Las infraestructuras de campo, la maquinaria de trabajo, el mantenimiento de los viñedos y los equipos para el control y seguimiento, se modifican o crecen con las necesidades y responden a las tecnologías más avanzadas del sector.




**D.1.** Distribución de ensayos en el C.T.T.V. “El Socorro”.

### *D.1.2. "El Encín"*

La finca "El Encín" está ubicada en la comarca del río Henares, entre las localidades madrileñas de Alcalá de Henares y Los Santos de la Humosa. Además incluye uno de los mejores bosques de ribera asociado a cantiles arcillosos de la Comunidad de Madrid. Esta finca consta de la mayor colección de variedades de la vid de Europa y dos viñedos experimentales de gran extensión. En uno de estos viñedos se llevó a cabo el estudio correspondiente al capítulo 6.

## D.2. Suelo

En las áreas de estudio se han registrado amplias diferencias entre los tipos de suelo. Ello es consecuencia del diferente grado de evolución que han sufrido los suelos que se han desarrollado sobre el aluvial del río Henares en el caso de la finca de "El Encín" y los aluviales de los ríos Tajo y Tajuña en el caso del centro de transferencia tecnológica "El Socorro".

Por su situación topográfica, se trata de zonas en las que los nuevos aportes de materiales son relativamente frecuentes, bien como resultado de las avenidas, o por el coluvionamiento de las zonas más inestables de las laderas. Lo primero es típico de cauces inadecuadamente regulados de una cierta entidad, mientras que lo segundo presenta una alta incidencia en las proximidades de los arroyos que completan la red de drenaje. En consecuencia, los suelos de fondos de valle o vega presentan una escasa evolución derivada de esta dinámica. Así, podemos encontrar suelos muy evolucionados pertenecientes al orden Alfisol, que han desarrollado potentes horizontes argílicos, con o sin

rubefacción, hasta suelos muy incipientes donde el grado de evolución es de moderado a bajo (Inceptisol y Entisol).

A continuación se detallan dos tablas explicativas de los suelos más característicos de las regiones vitivinícolas del Mundo (Tabla D.1) y los tipos de suelo en los viñedos muestreados en los trabajos que confieren esta Tesis (Tabla D.2)

**Tabla D.1.** Grandes grupos de suelo correspondientes a las grandes clases de suelo utilizados en la viticultura mundial (White, 2003).

| Clasificación de los suelos del Mundo utilizados en viticultura | | | |
|---|---|---|---|
| Grandes grupos de suelo | Características del perfil | Clasificación FAO-Unesco | Características principales |
| Litosoles | Sin perfil diferenciado | Leptosoles | Suelos débilmente desarrollados y poco profundos. Formación de depósitos aluviales por el movimiento del agua |
| Arenas calcáreas<br>Arenas silíceas<br>Suelos aluviales | | Fluvisoles | |
| Suelos calcáreos marrones y rojos | Perfil poco desarrollado, algunos con superficie oscura dada por la materia orgánica | Calcisoles | Acumulación de carbonato cálcico |
| Rendzinas | | Kastanzems | Suelos ricos en materia orgánica y colores marrones o pardizos |
| | | Phaeozems | Suelos ricos en materia orgánica y colores oscuros |
| Suelos pardos<br>Suelos marrones lixiviados<br>Solonetz solenizados | Suelos medianamente lixiviados (no muy ácidos), perfil diferenciado con doble textura | Cambisols | Cambios en color, estructura y consistencia |
| | | Solonetz | Suelos muy salinos, gran cantidad de iones de $Na^+$ en su estructura |
| Suelos rojos podzólicos<br>Suelos ocres podzólicos<br>Suelos marrones podzólicos | Acidez media-alta y alto contraste en la textura | Acrisoles<br>Luvisoles<br>Podzoluvisoles | Altamente ácidos y muy poco básico y acumulación de arcillas por percolación |
| Krasnozems<br>Terra Rossas<br>Suelos rojos<br>Suelos rojos calcáreos | Predominio de sesquióxidos | Ferralsoles<br>Plintosoles<br>Calcisoles | Suelos con alto contenido en sesquióxidos y arcillas multicolor que solidifican cuando se exponen a la interperie |

**Tabla D.2**. Suelos presentes en los áreas de estudio (El Encín y El Socorro) según Soil Taxonomy System (FAO, 1995)

| ORDEN | SUBORDEN | GRUPO | SUBGRUPO | EQUIVALENCIA FAO (1995) |
|---|---|---|---|---|
| Alfisoles | Xeralfs | Rhodoxeralfs | Típico | Luvisol crómico |
| | | Haploxeralfs | Vértico | Luvisol vértico |
| | | | Ácuico | Luvisol gleico |
| | | | Cálcico | Luvisol cálcico |
| | | | Típico | Luvisol háplico |
| Inceptisoles | Ochrepts | Xerochrepts | Vértico | Cambisol vértico |
| | | | Ácuico | Cambisol gleico |
| | | | Fluvéntico | Cambisol eutrico |
| | | | Calcixerólico | Cambisol calcárico |
| | | | | Calcisol háplico |
| | | | Típico | Cambisol eutrico |
| Entisoles | Fluvents | Xerofluvents | Típico | Fluvisol calcárico |
| | Psamments | Xeropsamments | Típico | Arenosol típico |

## D.3. Clima

El clima de una región viene determinado por una combinación de distintas variables meteorológicas siendo la temperatura y la precipitación los elementos principales. A continuación se expone una ampliación de los datos climatológicos del C.T.T.V. El Socorro y de la finca El Encín, obtenidos de sus respectivas estaciones meteorológicas durante los años de los estudios de esta memoria.

### *D.3.1. El Socorro*

Las tablas que se muestran son los datos meteorológicos obtenidos entre los años 2006 y 2008, en las inmediaciones de los viñedos muestreados en los capítulos 3, 4 y 5. Los datos indican las temperaturas máximas y mínimas de cada mes, así como las medias en grados centígrados (ºC) y los valores de precipitación media acumulada cada mes en milímetros (mm).

| 2006 | T.Máxima ºC. | T.Mínima ºC. | Lluvia mm. | T.Media Máx. | T.Media Mín. |
|---|---|---|---|---|---|
| **Enero** | 12,6 | -12,0 | 39,2 | 7,5 | -2,2 |
| **Febrero** | 13,6 | -7,0 | 41,2 | 8,1 | -0,7 |
| **Marzo** | 14,5 | -7,0 | 21,5 | 14,0 | -0,9 |
| **Abril** | 21,0 | -3,0 | 31,0 | 17,4 | 2,5 |
| **Mayo** | 32,5 | 1,5 | 16,0 | 23,2 | 5,1 |
| **Junio** | 34,0 | 4,5 | 38,0 | 29,3 | 10,9 |
| **Julio** | 36,0 | 8,1 | 2,2 | 35,7 | 10,2 |
| **Agosto** | 33,0 | 7,5 | 1,1 | 35,8 | 11,3 |
| **Septiembre** | 36,0 | 3,6 | 5 | 28,9 | 8,4 |
| **Octubre** | 27,1 | 3,0 | 92,8 | 22,6 | 8,5 |
| **Noviembre** | 19,2 | 0,5 | 89,1 | 14,6 | 3,4 |
| **Diciembre** | 14,0 | -7,5 | 20,5 | 8,7 | -1,9 |
| **Sumas** | **302,0** | **-6,0** | **397,6** | **245,8** | **54,6** |
| **Medias** | **25,17** | **-0,50** | **21,7** | **20,48** | **4,55** |

| 2007 | T.Máxima ºC. | T.Mínima ºC. | Lluvia mm. | T.Media Máx. | T.Media Mín. |
|---|---|---|---|---|---|
| **Enero** | 15,0 | -6,1 | 16,3 | 11,7 | -1,3 |
| **Febrero** | 20,0 | -1,4 | 39,8 | 13,0 | 2,7 |
| **Marzo** | 23,0 | -3,3 | 45,3 | 14,1 | 1,8 |
| **Abril** | 26,8 | -3,5 | 64,1 | 17,8 | 5,7 |
| **Mayo** | 28,0 | 3,1 | 54,2 | 21,5 | 6,8 |
| **Junio** | 35,5 | 8,7 | 39,5 | 28,2 | 14,8 |
| **Julio** | 37,8 | 10,3 | 1,8 | 34,8 | 11,2 |
| **Agosto** | 39,2 | 9,2 | 8,6 | 36,9 | 11,3 |
| **Septiembre** | 37,5 | 5,5 | 9,4 | 29,4 | 8,1 |
| **Octubre** | 26,7 | 2,0 | 42,5 | 17,8 | 5,1 |
| **Noviembre** | 22,6 | -6,6 | 18,7 | 15,6 | -2,4 |
| **Diciembre** | 16,2 | -11,0 | 11,9 | 9,9 | -8,5 |
| **Sumas** | **302,0** | **-6,0** | **352,1** | **250,7** | **55,3** |
| **Medias** | **25,17** | **-0,50** | **31,0** | **20,89** | **4,61** |

| 2008 | T.Máxima ºC. | T.Mínima ºC. | Lluvia mm. | T.Media Máx. | T.Media Mín. |
|---|---|---|---|---|---|
| **Enero** | 15,1 | -3,7 | 18,2 | 12,2 | 1,0 |
| **Febrero** | 17,2 | -9,2 | 42,7 | 14,4 | -3,1 |
| **Marzo** | 20.3 | -5,3 | 6,0 | 13,9 | -1,1 |
| **Abril** | 23,2 | 2,0 | 111,1 | 17,8 | 4,3 |
| **Mayo** | 25,4, | 2,3 | 117,1 | 21,1 | 3,8 |
| **Junio** | 32,9 | 4,5 | 31,5 | 27,3 | 9,9 |
| **Julio** | 37,5 | 8,0 | 14,6 | 32,5 | 9,5 |
| **Agosto** | 38,1 | 7,2 | 25,7 | 35,8 | 10,2 |
| **Septiembre** | 32,2 | 5,3 | 43,1 | 29,7 | 8,6 |
| **Octubre** | 23,7 | 0,0 | 81,3 | 19,0 | 4,3 |
| **Noviembre** | 17,0 | -6,0 | 9,4 | 13,7 | -4,3 |
| **Diciembre** | 13,5 | -7,6 | 49,5 | 10,3 | -4,2 |
| **Sumas** | **250,4** | **-2,5** | **550,2** | **247,7** | **38,9** |
| **Medias** | **25,04** | **-0,21** | **45,85** | **20,64** | **3,24** |

## *D.3.2. El Encín*

Los cálculos del periodo 2006-2009 de los valores climatológicos, obtenidos de la estación meteorológica de la finca de El Encín se muestran a continuación, de acuerdo a las temperaturas máximas y mínimas y sus respectivas medias, así como la precipitación media durante los años de muestreo.

| 2006 | T.Máxima ºC. | T.Mínima ºC. | Lluvia mm. | T.Media Máx. | T.Media Mín. |
|---|---|---|---|---|---|
| **Enero** | 13,0 | -12,0 | 39,2 | 6,5 | -2,6 |
| **Febrero** | 15,0 | -7,0 | 41,2 | 9,2 | -2,7 |
| **Marzo** | 19,5 | -7,0 | 21,5 | 13,0 | -0,9 |
| **Abril** | 23,0 | -3,0 | 31,0 | 17,7 | 2,1 |
| **Mayo** | 32,5 | 1,5 | 16,0 | 26,2 | 6,7 |
| **Junio** | 34,0 | 4,5 | 38,0 | 29,3 | 10,6 |
| **Julio** | 36,0 | 10,0 | 2,5 | 34,7 | 13,2 |
| **Agosto** | 33,0 | 7,0 | 5,6 | 32,2 | 12,3 |
| **Septiembre** | 36,0 | 3,0 | 16,6 | 26,7 | 8,4 |
| **Octubre** | 27,0 | 3,0 | 92,8 | 22,5 | 7,5 |
| **Noviembre** | 19,0 | 0,0 | 89,1 | 15,6 | 3,4 |
| **Diciembre** | 14,0 | -6,0 | 20,5 | 8,7 | -0,9 |
| **Sumas** | **302,0** | **-6,0** | **414,0** | **242,3** | **57,1** |
| **Medias** | **25,17** | **-0,50** | **23,5** | **20,19** | **4,76** |

| 2007 | T.Máxima ºC. | T.Mínima ºC. | Lluvia mm. | T.Media Máx. | T.Media Mín. |
|---|---|---|---|---|---|
| **Enero** | 15,0 | -8,0 | 11,4 | 7,7 | -1,3 |
| **Febrero** | 20,0 | -4,0 | 47,8 | 12,0 | 2,7 |
| **Marzo** | 20,0 | -4,0 | 45,4 | 14,6 | 1,8 |
| **Abril** | 25,0 | -3,5 | 84,7 | 16,8 | 5,6 |
| **Mayo** | 28,0 | 1,0 | 99,2 | 20,2 | 6,8 |
| **Junio** | 34,5 | 8,0 | 39,5 | 26,2 | 11,8 |
| **Julio** | 39,0 | 11,0 | 1,4 | 32,8 | 15,1 |
| **Agosto** | 40,0 | 7,0 | 8,4 | 32,2 | 14,6 |
| **Septiembre** | 33,5 | 4,0 | 8,9 | 28,0 | 12,4 |
| **Octubre** | 25,0 | 1,0 | 42,5 | 20,6 | 7,1 |
| **Noviembre** | 22,0 | -8,0 | 29,7 | 15,3 | -0,4 |
| **Diciembre** | 16,0 | -7,0 | 10,8 | 9,9 | -1,5 |
| **Sumas** | **302,0** | **-6,0** | **429,7** | **236,3** | **74,7** |
| **Medias** | **25,17** | **-0,50** | **38,5** | **19,69** | **6,23** |

| 2008 | T.Máxima ºC. | T.Mínima ºC. | Lluvia mm. | T.Media Máx. | T.Media Mín. |
|---|---|---|---|---|---|
| **Enero** | 18,0 | -7,0 | 28,9 | 11,7 | -0,9 |
| **Febrero** | 20,0 | -5,0 | 32,9 | 12,4 | -1,1 |
| **Marzo** | 21,0 | -8,0 | 0,0 | 14,9 | -0,7 |
| **Abril** | 25,5 | -2,0 | 95,8 | 17,2 | 4,3 |
| **Mayo** | 26,0 | 1,0 | 121,0 | 19,7 | 6,8 |
| **Junio** | 35,0 | 4,0 | 31,5 | 26,3 | 10,4 |
| **Julio** | 37,0 | 9,0 | 4,3 | 30,5 | 13,5 |
| **Agosto** | 37,0 | 11,0 | 3,2 | 31,8 | 13,2 |
| **Septiembre** | 30,0 | 5,0 | 27,1 | 24,7 | 10,6 |
| **Octubre** | 24,0 | -1,0 | 81,3 | 19,4 | 5,8 |
| **Noviembre** | 17,0 | -8,0 | 9,4 | 11,4 | -1,3 |
| **Diciembre** | 16,0 | -6,0 | 49,5 | 8,3 | -1,7 |
| **Sumas** | **306,5** | **-7,0** | **484,9** | **228,3** | **58,9** |
| **Medias** | **25,54** | **-0,58** | **40,41** | **19,03** | **4,91** |

| 2009 | T.Máxima ºC. | T.Mínima ºC. | Lluvia mm. | T.Media Máx. | T.Media Mín. |
|---|---|---|---|---|---|
| **Enero** | 13,5 | -13,0 | 59,1 | 5,9 | -2,0 |
| **Febrero** | 18,5 | -5,0 | 37,8 | 10,6 | -1,5 |
| **Marzo** | 25,0 | -0,5 | 42,1 | 18,6 | 2,1 |
| **Abril** | 26,0 | -2,5 | 37,6 | 19,1 | 3,9 |
| **Mayo** | 35,0 | 5,0 | 17,9 | 26,7 | 9,5 |
| **Junio** | 40,0 | 10,0 | 20,6 | 31,2 | 13,4 |
| **Julio** | 39,5 | 12,0 | 0,0 | 35,6 | 14,4 |
| **Agosto** | 38,0 | 13,0 | 5,1 | 34,9 | 16,3 |
| **Septiembre** | 34,0 | 6,5 | 17,6 | 27,8 | 13,1 |
| **Octubre** | 30,0 | 1,0 | 35,2 | 24,6 | 8,3 |
| **Noviembre** | 24,0 | -1,0 | 53,4 | 17,1 | 4,1 |
| **Diciembre** | 15,5 | -10,0 | 141,7 | 9,4 | -1,9 |
| **Sumas** | **339,0** | **15,5** | **468,1** | **261,5** | **79,7** |
| **Medias** | **28,25** | **1,29** | **39,01** | **21,79** | **6,64** |

# Apéndice E

# ESTADÍSTICA

# Apéndice E
# Estadística

## E.1. Leyenda de índices estadísticos

A continuación se describen los términos utilizados en los índices estadísticos empleados en esta memoria, además se da una explicación razonada sobre su uso y aplicación, siendo así de gran utilidad para futuros trabajos de Ecología.

| Término | Descripción |
|---|---|
| **D** | Denotación del índice de Simpson |
| **1-D** | Complemento de D en el índice de Simpson |
| $\Sigma^S$ | Sumatorio donde S es el número de especies |
| **H'** | Denotación del índice de Shannon-Wiener |
| $\log_2$ | logaritmo en base 2 |
| **p> ó p<** | índice de significación estadística |
| ***pi*** | proporción de muestra para la especie *i* |
| **S** | número o riqueza de especies |
| ***t*** | valor de t del estadígrafo *t*-de Student |

De acuerdo con Moreno et al., 2001, el **índice de Shannon-Wiener (H')** expresa la uniformidad de los valores de importancia a través de todas las especies de la muestra. Mide el grado promedio de incertidumbre en predecir a que especie pertenecerá un individuo escogido al azar de una colección. Además asume que los individuos son seleccionados al azar y que todas las especies están representadas en la muestra. Adquiere valores entre cero, cuando hay una sola especie, y el logaritmo de S, cuando todas las especies están representadas por el mismo número de individuos. La fórmula general para su cálculo es la siguiente:

$$H' = -\Sigma p_i \ln p_i$$

Para probar la hipótesis nula de que las diversidades provenientes de dos muestras son iguales, seguimos el siguiente procedimiento:

- Para cada muestra se calcula el índice de biodiversidad ponderado (Hp) en función de la frecuencia de cada especie:

$$Hp = \frac{(N \log N) - (\Sigma fi \log fi)}{N}$$

Donde (*fi*) es la frecuencia (número de individuos) registrados para la especie i.

- Para cada muestra se calcula la varianza del índice de biodiversidad ponderado:

$$\text{var} = \frac{\left[\Sigma f_i \log^2 f_i - (\Sigma f_i \log f_i)^2\right]/N}{N^2}$$

- Tras la obtención de la varianza, se calcula la diferencia de las varianzas de ambas muestras:

$$D_{\text{var}} = \sqrt{\text{var}_1 + \text{var}_2}$$

- Se obtiene el valor de *t*:
-

$$t = \frac{Hp_1 - Hp_2}{D\ \text{var}}$$

- Se calculan los grados de libertad asociados con el valor de *t*:

$$g.l. = \frac{(\mathrm{var}_1 + \mathrm{var}_2)^2}{(\mathrm{var}_1^{\,2}/N_1) + (\mathrm{var}_2^{\,2}/N_2)}$$

- Se busca en las tablas estadísticas el valor de la distribución asociada al valor de t para los grados de libertad calculados y con ello decidimos si aceptar o rechazar la hipótesis nula.

De este modo se obtendría el índice de biodiversidad para las especies dadas en esta Tesis. Además se empleó el índice de **Simpson (*D*)** para medir el grado de dominancia, su inverso representa, por tanto, la equidad. Manifiesta la probabilidad de que dos individuos tomados al azar de una muestra sean de la misma especie. Está fuertemente influido por la importancia de las especies más dominantes:

$$D = \Sigma^S (p_i)^2$$

Donde (*pi*) es la abundancia proporcional de la especie *i*, es decir, el número de individuos de la especie i dividido entre el número total de individuos de la muestra. Como su valor es inverso a la equidad, la diversidad puede calcularse como:

$$1 - D = 1 - \Sigma (p_i)^2$$

# E.2. Análisis de factores discriminantes (AFD)

El análisis discriminante puede considerarse una técnica multivariante de clasificación de individuos en la que se presupone la existencia de dos o más grupos bien definidos a priori y se persigue uno de los siguientes objetivos:

1. Describir las diferencias existentes entre esos grupos en base a los valores que toman ciertas variables sobre los individuos de cada uno de los grupos

2. Clasificar nuevos individuos en alguno de los grupos preexistentes en función de los valores que toman ciertas variables para esos individuos.

El análisis discriminante por pasos es una variante del análisis multivariante en el que las variables son añadidas a la función discriminante una a una, hasta que la adición de otra más no proporcione una discriminación significativamente mayor. De esta manera se consigue el número mínimo de variables con el que obtiene la mejor separación entre grupos. Una vez obtenidas las funciones discriminantes, en una de origen desconocido, bastará determinar las distintas variables utilizadas en la discriminación y sustituir los valores obtenidos en las funciones de clasificación (Peña Sánchez de Rivera, 2002).

**Tabla E.1.** Coeficientes estandarizados de dos funciones discriminantes canónicas obtenidas en la clasificación de las especies de levaduras aisladas en el estudio de los diferentes parámetros agronómicos analizados durante 2006, 2007 y 2008.

| Variables | Función 1 (86,7%)* | Función 2 (9,6)* |
|---|---|---|
| Convencional | 2,273 | -1,076 |
| Ecológico | 4,725 | -1,847 |
| Syrah | -4,192 | -7,687 |
| Garnacha | 5,282 | -4,539 |
| Barbera | -6,31 | 5,206 |
| Tempranillo | 2,936 | -0,123 |
| Laboreo | 0,600 | 4,429 |
| Herbicida | 0,791 | 2,458 |
| Cubierta | 0,185 | 0,842 |
| Azufre A | -2,225 | 2,784 |
| Azufre B | 2,932 | -2018 |
| Penconazol A | 0,805 | 3,906 |

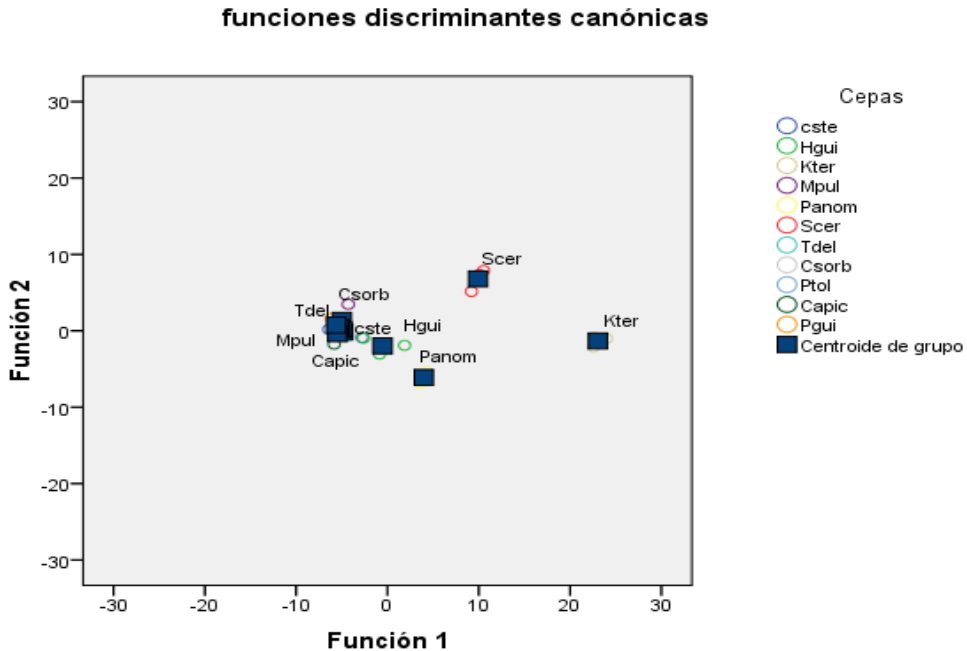
* Porcentaje de la varianza explicado

**Tabla E.2.** Clasificación [(a)] de las especies de levaduras aisladas durante los años 2006, 2007 y 2008, mediante la aplicación del análisis de funciones discriminantes por pasos en función de los parámetros agronómicos analizados. (Cste = *C. stellata*, Hgui= *H. guilliermondii*, Kter = *Kluyveromyces thermotolerans*, Mpul = *M. pulcherrima*, P. anom = *P. anomala*, Scer = *S. cerevisiae*, Tdel = *T. delbrueckii*, Csorb = *C. sorbosa*, Ptol = *P. toletana*, Capic = *C. apicola*, Pgui = *P. guilliermondii*).

| | Especies | Grupo de pertenencia pronosticado | | | | | | | | | | | Total |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Cste | Hgui | Kter | Mpul | Panom | Scer | Tdel | Csorb | Ptol | Capic | Pgui | |
| % | Cste | 66,6 | 0 | 0 | 0 | 0 | 0 | 0 | 33,3 | 0 | 0 | 0 | 100 |
| | Hgui | 33,3 | 66,6 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 100 |
| | Kter | 0 | 0 | 100 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 100 |
| | Mpul | 0 | 0 | 0 | 33,3 | 0 | 0 | 0 | 66,6 | 0 | 0 | 0 | 100 |
| | Panom | 0 | 0 | 0 | 0 | 100 | 0 | 0 | 0 | 0 | 0 | 0 | 100 |
| | Scer | 0 | 0 | 0 | 0 | 0 | 100 | 0 | 0 | 0 | 0 | 0 | 100 |
| | Tdel | 0 | 0 | 0 | 0 | 0 | 0 | 33,3 | 33,3 | 33,3 | 0 | 0 | 100 |
| | Csorb | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 100 | 0 | 0 | 0 | 100 |
| | Ptol | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 66,6 | 33,3 | 0 | 0 | 100 |
| | Capic | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 66,6 | 0 | 33,3 | 0 | 100 |
| | Pgui | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 66,6 | 0 | 0 | 33,3 | 100 |

a. Clasificados correctamente el 63,6% de los casos agrupados originales

**Figura E.1.** Representación gráfica de la clasificación de las especies de levaduras aisladas durante los años 2006, 2007 y 2008, en el plano formado por las funciones discriminantes canónicas obtenidas en función de diferentes parámetros agronómicos. . (Cste = *C. stellata*, Hgui= *H. guilliermondii*, Kter = *Kluyveromyces thermotolerans*, Mpul = *M. pulcherrima*, P. anom = *P. anomala*, Scer = *S. cerevisiae*, Tdel = *T. delbrueckii*, Csorb = *C. sorbosa*, Ptol = *P. toletana*, Capic = *C. apicola*, Pgui = *P. guilliermondii*).



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